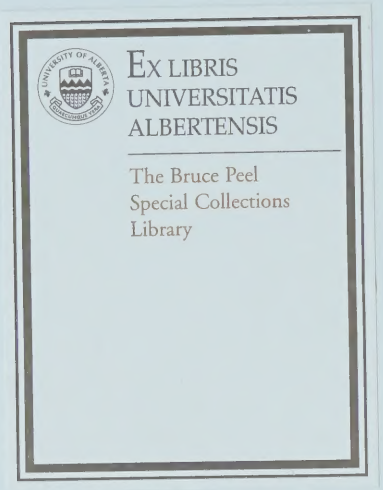
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The Role of the Medial Prefrontal Cortex in Fear and Anxiety-Related Behaviour

by

Akeel A. Shah

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Psychology

Edmonton, Alberta

Spring, 2005

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE ROLE OF THE MEDIAL PREFRONTAL CORTEX IN FEAR AND ANXIETY-RELATED BEHAVIOUR submitted by AKEEL A. SHAH in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dedicated to my parents, Muhammad and Tehzib Shah.

Abstract

Research investigating the role of the medial prefrontal cortex (MPFC) on the behavioural expression of fear/anxiety has been limited. In light of this, the present thesis systematically investigated the effects of direct manipulation of the MPFC on fear-related behaviour. The first study examined the effects of excitotoxic MPFC lesions on rats' behaviour in three well-validated models of fear/anxiety. MPFC lesions produced anxiolytic-like effects in the elevated plus-maze, shock-probe burying, and social interaction tests, thus providing evidence that the MPFC influences a variety of fear-related behavioural responses (Chapter 2). The second study examined the effects of bilateral intra-MPFC microinfusions (targeting the dorsal or ventral MPFC) of the indirect $GABA_A$ agonist midazolam on elevated plus-maze and shock-probe burying behaviour. Intra-MPFC infusions of midazolam (5 μg/0.5μl) produced anxiolytic effects in both tests (i.e. increased open-arm activity and decreased probe-directed burying), while the dorsal-ventral position of the MFPC infusion was not a significant factor (Chapter 3). The third study extended these findings by showing that intra-MPFC infusions of the direct $GABA_A$ agonist muscimol (0.175 nmol or 4 nmol/0.5 μl) also produced anxiolytic effects in both tests (Chapter 4). The findings from both studies suggest that MPFC $GABA_A$ receptors participate in the neural processes underlying fear behaviour. The fourth study examined the effects of intra-MPFC infusions of three DA subtype selective antagonists, SCH-23390 (D1), remoxipride (D2), and L-745,870 (D4), in the plus-maze and shock-probe tests. The D4 antagonist increased open-arm

activity (1 nmol/0.5 µl), and decreased the duration of burying (0.2 or 1 nmol/0.5 µl), while the D1 and D2 antagonists had no effects, suggesting that blockade of MPFC D4 receptors produces anxiolysis (Chapter 5). The final study examined the effects of the general MPFC DA receptor agonist apomorphine on fear behaviour. Intra-MPFC infusions of apomorphine were shown to increase open-arm activity (1 and 6 µg/0.5 µl), and attenuate burying (6 µg/0.5 µl) (Chapter 6), suggesting that stimulation of MPFC DA receptors is also capable of attenuating fear-related behaviour. Overall, the present findings from provide evidence that the MPFC, and specifically MPFC $GABA_A$ and DA receptors, are involved in the neural mediation of fear behaviour.

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List of Abbreviations

µg	microgram
µl	microlitre
µm	micrometre
AC	alternating current
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AP	anterior-posterior
cc	cubic centimetre
cm	centimetre
CS	conditioned stimulus
DA	dopamine
DV	dorsal-ventral
EPM	elevated plus-maze test
Fig.	figure
Fos-LI	fos-like immunoreactivity
GABA	γ-aminobutyric acid
h	hour
HPA	hypothalamic-pituitary-adrenal
im	intramuscular
ip	intraperitoneal
IQ	interquartile range
IU	international units

kg	kilogram
LOG	logarithmic transformation
LSD	least significant difference (post-hoc test)
mA	milliampere
mg	milligram
min.	minutes
ml	millilitre
ML	medial-lateral
mm	millimetre
MPFC	medial prefrontal cortex
NAc	nucleus accumbens
nmol	nanomole
PAG	periaqueductal grey
PBS	phosphate-buffered saline
po	peroral (oral administration)
PTZ	pentylenetetrazol
RTC	resistance to capture
S.E.M.	standard error of measure
SP	shock-probe burying test
vol	volume
VTA	ventral tegmental area
wt	weight

Chapter 1

Introduction

1. General Introduction

Empirical inquiry into the neural mechanisms underlying emotion is a relatively underdeveloped area of research within the neurosciences. For instance, both cognitive (e.g. learning and memory) and sensory processes (e.g. vision) have tended to receive more attention from neuroscientists than emotional processes. It has been argued that the paucity of research examining emotion is partially a product of how emotion is commonly conceptualized [78]. As a term in colloquial language, emotion typically refers to conscious experiences, or 'feelings', such as fear, anger, and happiness. As a result, past researchers have often been reluctant to specifically examine the neurobiological basis of emotion, since the conscious experience of emotion is inherently subjective and very difficult to operationally define. However, it has long been argued that an emotion consists of more than conscious experiences, with a set of behavioural and physiological responses being a crucial, and possibly pre-eminent, aspect of any emotion [61,77]. For example, in addition to a subjective experience of anger, this emotion is also defined by a set of responses such as offensive and/or threat-related behaviours and physiological changes such as increased blood pressure, heart rate, and corticosteroid release. Therefore, while it is true that the emotional experience of another human or animal cannot be directly observed, from an empirical standpoint emotion can be defined by the

behavioural and physiological responses that accompany these conscious experiences.

In recent years, research into the neurobiology of emotion has expanded and developed considerably as a result of the establishment of empirically validated animal models of emotion and the introduction of neuroimaging techniques. In particular, a great deal of progress has been made with regard to our understanding of how the brain mediates fear and anxiety-related processes [76,78,80,92]. Before fear and anxiety are discussed further it would be useful to define these emotional states. In general, both terms refer to the set of behavioural, physiological, and phenomenological responses arising from the perception of danger to an organism. These responses are adaptive and vital to survival of all mammals. This set of responses includes increases in heart rate, blood pressure, piloerection, release of glucocorticoids, behaviour such as fleeing or freezing, and of course the feeling of fear. When fear and anxiety are used as distinct terms, 'fear' is typically used to refer to emotional responses resulting from immediate threats, while 'anxiety' is typically used to refer to the persistence of these emotional responses beyond the existence of an immediate threat [3]. In addition, 'fear' is sometimes used to refer to 'normal' behaviour, while anxiety is taken to represent the pathological expression of this emotion. Stress is another important term which is often used in conjunction with fear and anxiety. Stress has traditionally been used to specifically represent the physiological reactions to a threatening situation. When compared to fear and anxiety, stress is both a more specific (focused on physiological responses) and a

more general term (since these responses are also crucial aspects of other emotions such as anger). While fear, anxiety, and stress have different connotations, for the purposes of the present paper all three terms will be used to represent responses specifically evoked by the presence or imminence of a potentially harmful stimulus (potential harm = threat).

When compared to other types of emotion, fear and anxiety have been argued to be particularly amenable to empirical inquiry [78]. Fear is likely one of the most basic and phylogenetically ancient emotions. As a result, there exists analogous fear-related behaviour which is similarly expressed, and readily recognizable as fear behaviour, across a variety of animal species (e.g. escaping from a pain-inducing stimulus). In addition, a number of animal models of anxiety have been developed and shown to have excellent predictive validity with regard to detecting anxiolysis [146] (these behavioural assays of fear and anxiety will be discussed in greater detail later in the introduction).

Another significant reason for conducting research on the neural basis of fear and anxiety is the role these emotional processes play in a variety of mental illnesses. Anxiety disorders, generally defined as the abnormally excessive expression of fear-related cognition and behaviour, include some of the most common types of mental disorders [68,123], causing considerable distress in large segments of the population. The DSM-IV recognizes several different types of anxiety disorders including generalized anxiety disorder, obsessive compulsive disorder, specific phobias, social phobia, panic disorder (with or without agoraphobia), agoraphobia (without panic disorder), and post-traumatic

stress disorder [1]. Lifetime prevalence for panic disorder, specific phobias, social phobia, obsessive compulsive disorder and post-traumatic stress disorder has been estimated to be 3.5%, 11.3%, 13.3%, 2.5%, and 7.8% respectively [68,69,87,122]. Estimates of the lifetime prevalence rates for all anxiety disorders combined have varied from approximately 11.2% to 24.9% in the general population [5,68]. In the United States alone anxiety disorders are estimated to affect over 15 million people a year and have an economic toll of over 40 billion dollars a year [44]. Improving our understanding of how the brain mediates fear/anxiety may also lead to improved treatment of other types of abnormal behaviour. Anxiety disorders are linked to other forms of mental illness, having high rates of co-morbidity with affective disorders such as depression. The lifetime prevalence for depression has been estimated to be as high as 50% for people receiving an anxiety disorder diagnosis [13].

2. Animal Models of Fear/Anxiety

2.1. Animal Models of Fear and Anxiety

Animal models of anxiety have typically strived to represent some aspect of the etiology, symptomatology, or treatment of human anxiety disorders [146]. The primary impetus for the development of animal models of anxiety has been for assessing the anxiolytic properties of various pharmacological compounds

(i.e., "screening" tests). In addition to the screening of potential anxiolytic compounds, animal models of anxiety have been very useful as a way of studying the general neural mechanisms underlying the expression of fear and anxiety-related behaviour [92,93,146]. In particular, when the use of animal models of anxiety have been combined with intracranial microinfusions of agonists/antagonists with selectivity for specific types of neurotransmitter systems (e.g., dopamine receptor antagonists), these models have been useful in mapping out brain regions and neurochemical processes influencing fear and anxiety [92]. Animal models of anxiety have primarily utilized behavioural responses to fear/anxiety-provoking stimuli, since physiological responses are typically not unique to a particular emotion. (e.g. several different types of emotion might be mistaken for fear since similar physiological arousal responses are often expressed across different emotions).

There are several different issues involved in the validation of anxiety models. Arguably the most important concern is establishing the predictive or correlational validity of the model. Models with predictive or correlational validity must be capable of accurately detecting the anxiolytic effects of a specific drug treatment known to be clinically effective. The therapeutic treatment which has been most commonly used to establish the predictive validity of animal models of anxiety has been treatment with benzodiazepines [125,146]. Homological validity is another important criterion for establishing the usefulness of a particular model. A model is said to be homologous if the fear-provoking stimulus used in the animal model is capable of producing a

fear/anxiety-related response in humans. Another criterion for evaluating the validity of anxiety models is isomorphism. A model is said to be isomorphic if the fear/anxiety response in the animal model is similar to a fear/anxiety response expressed in humans. It has been argued that homology and isomorphism are important in establishing the validity of any animal model of anxiety since it seems to be a reasonable requirement that some functional similarity exist between human fear/anxiety responses and the animal model [142,146].

Several different behavioral models of fear/anxiety have been developed, each with its own distinctive attributes. One way in which these different anxiety models have been categorized is whether they primarily examine a conditioned or unconditioned behavioural response. Examples of unconditioned fear/anxiety paradigms are the elevated plus-maze, mouse defense test battery, social interaction test, light/dark exploration, and open field tests. Examples of conditioned fear paradigms include conditioned freezing, fear-potentiated startle, conflict, and shock-probe retention tests [125]. The following section will describe three behavioural assays of anxiety used in the present thesis: the elevated plus-maze, shock-probe burying, and social interaction test.

2.2. Elevated Plus-Maze Test

At present, the elevated plus-maze is one of the most extensively utilized animal models of anxiety [52]. The elevated plus-maze is a plus-shaped

apparatus, typically of wooden or polymer construction. It consists of two 'open' arms (arms without surrounding walls), and two closed arms (arms with walls on both sides), with all arms having an open roof. All arms are elevated above the floor (50 cm is a standard elevation; See Figure 1.1). During this test, which typically lasts for five minutes in duration, rats are allowed to explore the maze freely. Rats naturally avoid the open arms of the maze and will spend most of their exploration time confined to the closed arms [105,106]. The aversive nature of the open arms of the elevated plus-maze may primarily be due to a fear of open spaces leading to thigmotaxic behaviour (i.e. increased entries and time in closed arms) [149]. Forced exposure to the open arms increases physiological and behavioural markers of stress including the plasma corticosterone levels, defecation, and freezing behaviour [105]. An anxiolytic-like behavioural profile in this test is represented by an increased percentage of time spent in, and percentage of entries into, the open arms (i.e. increases in the following two ratios: total duration time spent in the open arms / total duration of time in any of the arms; number of open-arm entries / number of closed-arm entries) [105,106]. Benzodiazepine anxiolytics, such as diazepam and chlordiazepoxide, have been shown to increase both measures of open-arm activity, while fear-inducing, or 'anxiogenic' compounds, like yohimbine or beta-carbolines, have been shown to decrease the same measures [2,48,65,66,81,107]. Mixed anxiolytic-antidepressant compounds, such as alprazolam, have also been shown to produce increased open-arm activity [36,45,112].

2.3. Shock-Probe Burying Test

The shock-probe apparatus is a transparent plastic chamber, with the floor evenly covered with bedding material. An electrified probe is attached to and extends from one side of the chamber, elevated slightly above the bedding. Rats are allowed to explore the apparatus freely and typically make sufficient contact with the probe to receive an electric shock within the first minute. After receiving an electric shock from the probe, rats characteristically exhibit focused burying behaviour, spraying bedding material directly onto or towards the probe with rapid alternating movements of the forepaws (See Figure 1.2) [110]. This burying response can be elicited by a single electric shock, and has been argued to be a part of the rat's natural defensive repertoire [138]. The primary index of anxiety in this test is the total duration of probe-directed burying, with a decrease in burying behaviour representing anxiolysis [152]. Several studies have shown that known anxiolytic compounds, such as chlordiazepoxide, diazepam, and buspirone, reduce the duration of burying behaviour (e.g. [4,16,30,82,126,143,144,147,152]). It has also been shown that plasma levels of corticosterone and adrenaline are elevated during shock-probe testing, and these physiological stress responses can also be antagonized by the administration of benzodiazepine anxiolytics [24,72,130].

2.4. Social Interaction Test

The social interaction test examines the social behaviour of conspecific rodents. Various environmental factors are used to modify the baseline levels of anxiety in the test animal (high/low levels of lighting; unfamiliar/familiar setting) [31]. The duration of active social interaction (e.g. conspecific grooming, sniffing, climbing over) is the primary measure in this test, with an anxiolytic profile represented by an increase in social interaction. A number of studies have shown anxiolytics, such as diazepam, flurazepam, and buspirone, increase the duration of social interaction, while anxiogenic compounds such as beta-carbolines have been shown to decrease this measure [31-35].

3. The Role of the Prefrontal Cortex in Fear and Anxiety

3.1. Background

A variety of neural structures have been shown to have critical roles in mediating the behavioural and physiological responses that define fear and anxiety. Several brainstem structures are important in this regard. For example, the ventral region of the periaqueductal grey (PAG) is an important structure for the expression of freezing, while the parabrachial nucleus and dorsal PAG is known to affect heart rate and respiration [28,49,67]. Other areas of the brainstem such as the ventral tegmental area (e.g. attention, vigilance), nucleus reticularis pontis oralis (e.g. startle response), and nucleus ambiguus (e.g.

defecation) also influence fear and anxiety-related responses [76]. In the diencephalon, the hypothalamus is known to regulate fight/flight behavioural patterns and to control the release of stress-related hormones (i.e. corticosteroids) via the hypothalamic-pituitary-adrenal (HPA) axis [9,94]. While these 'lower level' structures generally play a role in the expression of specific fear-related responses, other brain regions are typically believed to be central structures in the overall integration and organization necessary for the full expression and experience of an emotion. From the time Paul MacLean shaped our conceptualization of the 'emotional brain', limbic structures have commonly been viewed as the primary brain regions underlying the neural mediation of emotion [84-86]. Limbic structures such as the hippocampus and the septum have been shown to be necessary for the expression of fear and anxiety in a variety of animal models of anxiety. For example, permanent or reversible septal and ventral hippocampal lesions have been shown to attenuate open-arm activity in the elevated plus-maze, and burying behaviour in the shock probe test [25,148,150]. Intra-septal or intra-hippocampal microinfusions of a variety of anxiolytics, such as midazolam, have also been shown to dramatically reduce fear and anxiety [92]. In light of this evidence, it has been proposed that the septum and hippocampus, acting in concert, play a critical role in the control and coordination of fear-related responses [42,43].

Historically, the amygdala has been the limbic structure that has garnered the most attention from researchers interested in the neurobiology of anxiety. The amygdala has been argued to be the central structure coordinating the

repertoire of responses that constitute fear and anxiety, and in the acquisition and expression of learned fears [78-80]. A vast amount of empirical evidence has been gathered to demonstrate the importance of the amygdala in mediating fear-related processes [19,78,79,113,120]. For example, neuroimaging work in humans has linked the amygdala to a number of anxiety disorders [120]. The findings from animal lesion studies have shown the amygdala to be critical for the learning of conditioned fear behaviours, where researchers have mapped out the involvement of various amygdaloid nuclei in fine detail [18,78,80].

Overall, it seems apparent that much of the recent research into the neurobiology of fear and anxiety has focused on amygdala, often presuming the region to be central in the neural mediation of fear-related responses. However, some findings have suggested that the amygdala may not be a crucial region for mediating all behavioural responses connected to fear and anxiety. For example, a number of studies have shown that amygdala lesions do not significantly affect rats' unconditioned fear responses, such as open-arm activity in the elevated plus-maze [70,131,148,151]. In addition, several studies have shown amygdala lesions have no apparent effect on the duration of burying behaviour in the shock-probe test [71,127,145,148,151]. Amygdala lesions have also been reported to have no behavioural effects in two other animal models of anxiety, the social interaction and successive alleys tests [91]. These findings suggest that the amygdala may not be critical for the neural processing of unconditioned fear responses; instead, the septum or hippocampus may play a more substantial role in these unconditioned behaviours. More generally, the results raise the

question of whether other brain regions have been overlooked due to the inordinate focus on the amygdala in research on the neurobiology of fear and anxiety.

One particular brain region that has received relatively little attention with respect to its role in mediating fear behaviour is the medial prefrontal cortex (MPFC). Although there is a great deal of evidence to suggest the MPFC may be an important structure for the neural mediation of anxiety-related physiological responses (e.g., increased corticosteroids), research specifically examining the influence of the region on anxiety-related behaviour has been limited. The following section will provide a brief overview of the anatomy and neurochemistry of the MPFC. Evidence suggesting the involvement of the MPFC in fear-related physiological responses, and the neurochemical correlates of anxiety, will also be discussed.

3.2. The Prefrontal Cortex: Neuroanatomy, Neurochemistry, and Physiological Fear Responses

The frontal cortex is commonly conceived of as being made up of three major areas: the motor cortex, the premotor cortex and the prefrontal cortex. The traditional anatomical criterion for the prefrontal cortex was defined by Rose and Woolsey as the region of the frontal cortex which receiving projections from the dorsomedial nucleus of the thalamus. According to this criterion, an analogous prefrontal region exists across a wide variety of mammalian species, including

rats [128]. However, at present there is some dispute as to the usefulness of this criterion in defining the prefrontal cortex, and other attempts to provide a single anatomical or functional criterion are still being elaborated [46,154,155]. Nevertheless, the prefrontal cortex is typically divided into the dorsolateral cortex, orbital frontal cortex, and medial prefrontal cortex (MPFC). It receives input from, and sends output to, a wide range of brain regions from the brainstem to the cortex. As such, it is often viewed as a center for the integration of various sensory inputs, and the executive planning necessary for 'higher-level' or complex behaviour [38,46]. The MPFC in rats is composed of the anterior cingulate, prelimbic, and infralimbic areas [46]. While the rat infralimbic region has a primitive lamination pattern and may not be innervated by the mediodorsal thalamus [163], it is typically included as a subregion of the MPFC [155]. The medial precentral area (also referred to as frontal area 2) is sometimes included as a MPFC region. However, it is often viewed as primarily a premotor or supplementary motor region and therefore will not be of focal interest in the present thesis [163].

The prefrontal cortex has substantial afferent and efferent connections with sensory, motor, autonomic and limbic regions of the brain [46,103,163]. The MPFC region of rodents and primates shows extensive connectivity with a variety of brainstem and midbrain nuclei, as well as limbic structures, that are believed to mediate the behavioural and physiological components of fear and anxiety (See Figure 1.3 for an overview of these connections). In the rat, there are efferent connections from the MPFC to the central and lateral nuclei of the

amygdala [56,90,129], and the amygdala in turn sends projections to the infralimbic and prelimbic regions of the MPFC [108]. The prelimbic and infralimbic regions also receive projections from the hippocampus, with the densest projections originating from the ventral hippocampus [62,134]. The MPFC, especially the infralimbic region, also sends projections to the lateral septum and the bed nucleus of the stria terminalis [157]. The prelimbic region of the MPFC sends extensive projections to the nucleus accumbens [157]. The MPFC is also one of the few cortical regions to have connections with the hypothalamus. The MPFC, particularly the prelimbic and infralimbic regions, projects to the hypothalamus, and there are also reciprocal connections from the hypothalamus to the MPFC [9,56,129,157]. Finally, the MPFC also communicates with mesencephalic, brainstem, and spinal areas thought to be important for the expression of some of the behavioural and physiological components of fear and anxiety, areas such as the PAG, parabrachial nucleus, nucleus of the solitary tract, motor nucleus of the vagus, nucleus ambiguous, and the spinal cord [56,104,129,139,156,157].

Given these anatomical relationships it is apparent that the MPFC could directly modulate fear/anxiety-related autonomic and neuroendocrine responses via its direct connections to a variety of diencephalic, brainstem, and spinal areas, or indirectly via its projections to limbic areas, such as the amygdala, that also modulate these processes. In either case, direct manipulation of the MPFC shows that it modulates specific autonomic and endocrine responses. For example, stimulation and/or lesioning of the MPFC has been reported to alter

various autonomic responses such as gastric motility, heart rate, blood pressure, temperature, piloerection and respiration [156]. With respect to endocrine function, stimulation of the MPFC has been shown to increase plasma levels of corticosterone [29], and lesions of the MPFC appear to decrease baseline and stress-induced levels of corticosterone, and susceptibility to stress-induced ulceration [132]. Other research suggests that the MPFC may be involved in feedback inhibition of the HPA stress response [26,156]. In light of the anatomical and functional evidence that exists, the MPFC, specifically the infralimbic and ventral prelimbic regions, are often thought of as being part of the limbic system [15,111,121,155]. The ventral MPFC, particularly the infralimbic region, has also been described as a 'visceral motor' area (i.e. a critical region for the integration of autonomic responses), because of its connections to autonomic brainstem nuclei and its ability to influence the autonomic function [56,139,156,157].

The prefrontal cortex receives substantial innervation from dopaminergic (VTA), serotonergic (raphe nuclei), noradrenergic (locus coeruleus), and cholinergic (basal forebrain) neurons [38,124]. The prefrontal cortex, like other cortical regions, also has very large numbers of γ-aminobutyric acid (GABA) interneurons [38]. Of particular interest are the dopamine (DA) projections from neurons in the VTA, since a substantial body of evidence has shown that this mesoprefrontal DA pathway is specifically active during periods of fear and stress. It has been reported that fear and stress-inducing stimuli such as restraint stress, inescapable foot-shock, tail pinch, and food deprivation, all increase

extracellular DA levels, or DA turnover, in the MPFC of rodents, with small or no changes in other regions receiving VTA DA innervation such as the nucleus accumbens, striatum, and even the amygdala [10-12,51,59,137,140]. Other fear-inducing stimuli such as predatory odours, social defeat stress, and systemic administration of anxiogenic drugs have also been shown to increase MPFC DA activity [6,101,135,141]. In addition, increases in MPFC DA activity provoked by fear and/or stress can be blocked by acute or chronic treatment with anxiolytic compounds, particularly $GABA_A$ agonists such as benzodiazepines [20,22,23,58,136]. Intriguingly, studies have also demonstrated that rats exposed to stressors show decreased levels of $GABA_A$ and benzodiazepine receptor binding in the prefrontal or frontal cortex [47,53]. Despite the evidence indicating MPFC DA, and to a lesser extent MPFC GABA, are involved in fear and anxiety, very few studies have *directly* examined the role of MPFC DA or GABA on fear and anxiety-related behaviour.

3.3. The Medial Prefrontal Cortex and Fear-Related Behaviour

There is considerable evidence to suggest that the prefrontal cortex, including the medial prefrontal region, is an important structure in the neural mediation of working memory, reward, addiction, cognitive flexibility, attention, and temporal ordering [7,38,39,115,116,153]. For example, a number of studies have shown that lesioning or neurochemical manipulation of the MPFC can

impair cognitive flexibility, working memory and reward (e.g. [14,116,118,119,153]). In contrast, there are relatively few studies that have directly and systemically examined the contribution of the MPFC in the neural processing of fear behaviour.

Neuropsychology has provided an abundance of anecdotal reports suggesting that the prefrontal cortex, and particularly the orbitomedial regions of the prefrontal cortex, influences affective behaviour [17,38]. However, the conclusions that can be drawn from neuropsychological findings are limited due to the quasi-experimental nature of these reports and the fact that lesions produced by brain injury are rarely confined to a specific region. Imaging studies have revealed that patients with specific anxiety disorders (such as obsessive-compulsive disorder, post-traumatic stress disorder, and social anxiety) show abnormal patterns of activity in the prefrontal cortex [89,120]. While these findings suggest that the prefrontal region contributes to the neural processing of fear and anxiety, these studies are limited since they only provide correlational, rather than causal evidence of the involvement of the prefrontal region in fear and anxiety (i.e. they cannot demonstrate that the region is necessary or sufficient for the neural mediation of fear and anxiety). In order to examine causal relationships, within both ethical and methodological constraints, many researchers have relied on experimental designs using infra-human subjects. However, as indicated below, this line of research has been problematic and rather limited in scope.

Despite the extensive evidence that exists in the animal literature suggesting MPFC involvement in the autonomic, endocrine, and neurochemical correlates of fear and anxiety, studies examining the behavioral effects of MPFC lesions have been inconsistent and sometimes contradictory in their findings. Since the publication of findings suggesting that MPFC lesions specifically impair extinction of conditioned freezing [78,97], much of the research examining the role of the MPFC in fear-related behaviour has centered around the extinction and inhibition of conditioned fear [102,113]. However, the findings regarding MPFC involvement in fear extinction have not been uniform. While some studies have replicated the initial findings [96,98,100], other studies have reported that MPFC lesions, particularly those targeting dorsal areas, do not affect the rate of within-session extinction [95,114,158]. Furthermore, various studies have reported both increased and decreased freezing behaviour during the acquisition and/or expression of conditioned freezing [75,95,158]. Additionally, a study examining conditioned fear behaviour in the fear-potentiated startle paradigm reported that MPFC lesions did not affect the expression, acquisition, or extinction of the conditioned startle response [40]. Other studies investigating the effects of MPFC lesions on a variety of aversive conditioning tasks have indicated impairment [37,63,83], enhancement [64], or no effect [54,60,74] on performance. This inconsistent pattern of results is further complicated by the possible mnemonic functions of the MPFC. Given the evidence indicating the MPFC influences various memory processes (e.g.[38,116,117,161,162]), it is possible that the effects on conditioned fear responses are a result of memory

dysfunction rather than a specific effect on fear-related behaviour. There are studies that have examined the effects of lesions on unconditioned fear behaviour, but they also have yielded an inconsistent pattern of findings. Lesions of the MPFC have been reported to increase [54,55,63] and decrease [74,75] fear-related behaviour as assessed by open field test. In the elevated plus-maze, MPFC lesions have been reported to produce anxiolytic-like effects [75,83,133], while other studies have reported MPFC lesions produce anxiogenesis, or no significant effect on open-arm activity [63,74].

Very few studies have directly investigated the role of the different MPFC neurotransmitter systems on fear behaviour. Dopamine is of particular interest because of the extensive evidence showing that mesoprefrontal DA is activated by fear, stress, and anxiety [10-12,51,59,137,140]. Since there is evidence to suggest that MPFC $GABA_A$ receptors may also be involved in fear and stress [47,53], and stimulation of the $GABA_A$ receptor complex is a very well-established means of producing anxiolysis, GABA is another neurotransmitter of interest. Excluding the findings of the present thesis, there is no published research examining the effects of intra-MPFC microinfusions of direct or indirect $GABA_A$ agonists on fear behaviour. There are a few studies that have investigated the effects of intra-MPFC microinfusions of DA agonists/antagonists on fear behaviour. However, the findings from these studies have not been consistent and are limited in scope. Intra-MPFC infusions of the DA receptor agonist apomorphine was reported to produce a pro-conflict effect, while the DA receptor antagonist cis-flupenthixol produced an anti-conflict

effect, suggesting an anxiogenic profile was produced by DA receptor stimulation and an anxiolytic profile was produced by DA receptor blockade [8]. Intra-MPFC infusions of these two DA drugs produced a similar anxiogenic and anxiolytic profile during pentylenetetrazol (PTZ) discrimination testing, but the drugs had no effect on chlordiazepoxide discrimination [7]. However, since both the conflict test and the PTZ discrimination test depend upon on learning and reward, it is difficult in these tests to distinguish effects on fear/anxiety from effects on learning and reward. More recent research found that intra-MPFC infusions of the D2 antagonist sulpiride attenuates fear-related behaviour in the elevated plus-maze, while the D2 agonist quinpirole had no significant effect on these behaviours [160]. On the other hand, the same group reported that intra-MPFC infusions of the D1 agonist SKF-81297 actually produced an anxiolytic profile in the elevated plus-maze [159]. Finally, intra-MPFC infusions of the DA agonist d-amphetamine, and the DA antagonist cis-flupenthixol, were both shown to attenuate expression, but not acquisition, of conditioned freezing, suggesting both drugs are capable of producing anxiolysis [109]. None of these intra-MPFC studies examined the effects of intra-MPFC infusions of selective D4 receptor agonists/antagonists on fear behaviour, despite evidence suggesting that D4 receptors influence unconditioned fear responses [27]. Since these intra-MPFC DA studies present an inconsistent overall pattern of results, additional research appears to be necessary to establish the effects of MPFC DA receptor stimulation/blockade on fear behaviour.

In light of these issues, and the paucity of studies directly investigating the influence of the different neurotransmitter systems that operate within the MPFC, it is clear that further research is necessary to definitively establish the role of the MPFC in the neural mediation of fear behaviour. In order to elucidate this role, the research described in the present thesis will examine the effects of lesions and neurochemical manipulation of the MPFC in several different animal models of fear and anxiety.

4. Thesis Outline and Research Objectives

The corpus of findings regarding the effects of MPFC lesions on fear and anxiety-related behavior has yielded an inconsistent body of findings. Studies have reported enhancement, attenuation, and no effect of MPFC lesions on a variety of fear-related behavioural responses [41,54,55,63,74,75,83,133]. In light of these diverse findings, the first study examined the effects of excitotoxic lesions of the MPFC in three well-validated behavioral assays of anxiety: the elevated plus-maze, social interaction and the shock-probe burying tests.

Despite considerable evidence that MFPC DA and GABA systems are involved in fear and anxiety [6,10-12,20-23,47,51,53,57-59,99,100,136,137,140,141], relatively few studies have directly examined the role of MPFC DA or GABA receptors on fear behaviour. To address this deficiency, the remaining studies were generally intended to explore the

behavioural effects of direct neurochemical manipulation of the MPFC. Specifically, no research has examined the effects of stimulation of the MPFC $GABA_A$ receptor complex on fear-related responses. Therefore, the second study examined the effects of intra-MPFC microinfusions of midazolam, an indirect $GABA_A$ receptor agonist, on behavioural responses in the elevated plus-maze and shock-probe burying tests. In addition, there are functional and anatomical data indicating that a dorsal-ventral distinction may exist within the MPFC [50], and dissociations between dorsal and ventral MPFC regions with respect to fear-related behaviour have been previously noted [63,73,83,95]. Therefore, the second study also compared the effects of midazolam infusions targeting the dorsal or ventral regions of the MPFC.

The third study extended the findings of the second by examining the effects of intra-MPFC infusions of a direct $GABA_A$ receptor agonist, muscimol, on fear behaviour. There were two principal reasons for conducting this study. The first was to examine the effects of directly activating $GABA_A$ receptors on fear behavior in the elevated plus-maze and shock-probe burying tests. The second was based on the fact that relatively high doses of muscimol have also been utilized to produce 'reversible lesions' which can be valuable in assessing the immediate effects of inactivation of a brain region [88]. Therefore the present study employed both a high and a low dose of muscimol.

As described earlier, it is known that a broad range of fear-inducing stimuli selectively increase the activity of VTA dopaminergic neurons projecting to the MPFC [10-12,51,59,137,140]. In addition, the increase in mesoprefrontal DA

activity in response to fear, anxiety, and stress has also been shown to be blocked by acute or chronic treatment with anxiolytic compounds [20,22,23,58,136]. Given the apparent relationship between fear-inducing stimuli and mesoprefrontal DA activity, it would be expected that directly stimulating or blocking MPFC DA receptors should produce clear effects on fear-related behavior. Both D1-like and D2-like DA receptors subtypes are found in the MPFC, and the D4 receptor subtype is estimated to be the most common D2-like receptor subtype in the prefrontal cortex [73]. Accordingly, the fourth study examined the effects of intra-MPFC infusions of three different DA antagonists selective for a particular DA subtype (either D1, D2, or D4) on fear behaviour in the elevated plus-maze and shock-probe burying tests. It was hypothesized that one or more of these antagonists would produce anxiolytic effects. In order to further investigate the influence of MPFC DA on fear behaviour, the final study examined the effects of MPFC DA receptor stimulation. Previous studies examining the effects of intracranial infusions of a variety of DA agonists have reported inconsistent findings [7,8,109,159]. Therefore, the fifth study systematically examined the effects of intra-MPFC infusions of a DA agonist, apomorphine, on behavior in the elevated plus-maze and shock-probe burying tests.

Figure 1-1.

Two pictures of rodents exploring different elevated plus-mazes.

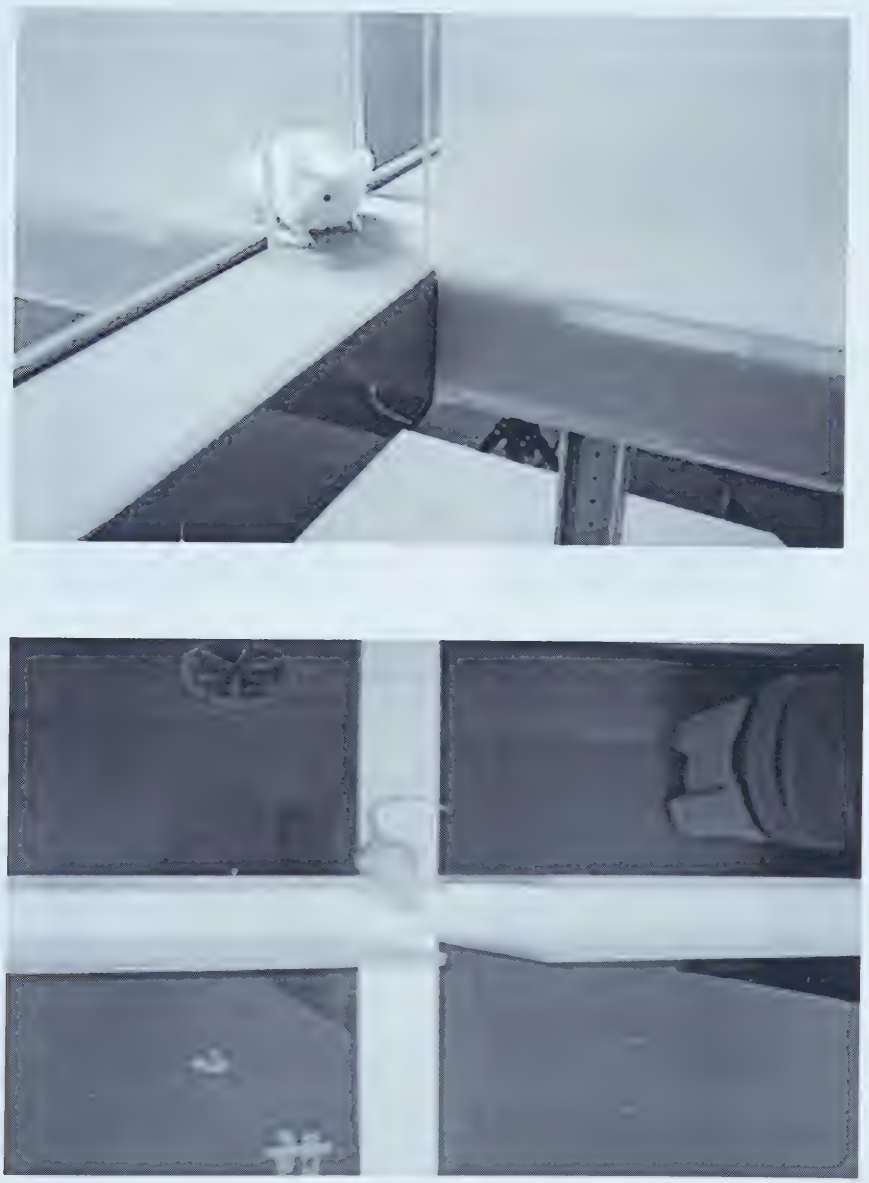


Figure 1-2.

This figure illustrates the basic components of the burying response observed during the shock-probe burying test:
A) The rat contacts the probe and an electric shock is administered. For a brief period afterwards the rat typically exhibits freezing behaviour. B) and C) These pictures illustrate the burying response. The rat uses alternating movements of the forepaws to spray bedding material onto the shock-probe. D) The probe has been covered completely with the bedding material.

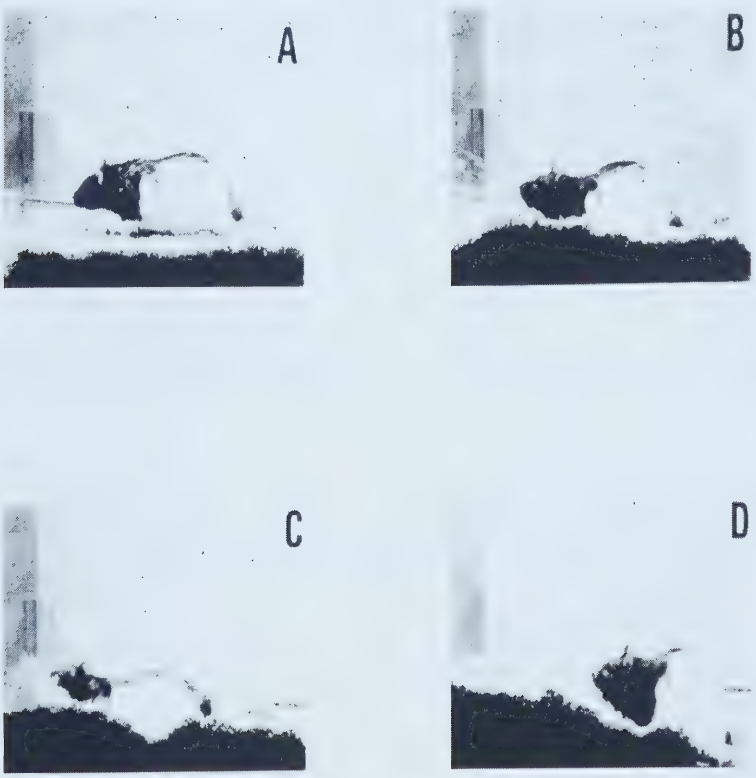
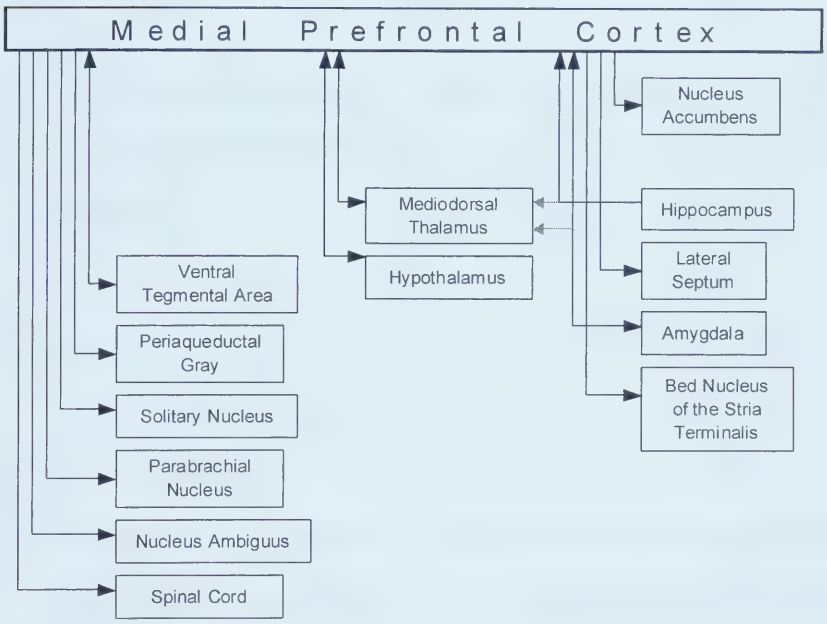


Figure 1-3.

Diagram illustrating selected efferent and afferent connections of the medial prefrontal cortex.



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Chapter 2

Excitotoxic Lesions of the Medial Prefrontal Cortex Attenuate Fear Responses in the Elevated Plus-Maze, Social Interaction, and Shock-Probe Burying

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1. Introduction

The prefrontal cortex is believed to have a role in a wide variety of processes including attention, reward, memory, inhibition, temporal ordering, and the integration of sensory information [5, 17, 50, 60]. In addition to these functions, a diverse but converging body of research has suggested that the medial prefrontal cortex (MPFC), which in the rat brain includes the prelimbic, infralimbic, and the anterior cingulate subregions, is involved in mediating the physiological responses to fear-inducing stimuli. It has been shown that the region is involved in fear-related endocrine and autonomic responses. For example, lesions of the rodent MPFC have been shown to affect susceptibility to stress-induced ulceration [23, 63, 65] and to decrease peak stress-induced corticosterone levels [63]. The MPFC has also been shown to modulate the hypothalamic-pituitary-adrenal (HPA) axis by acting as a negative-feedback site [11]. In addition, stimulation and/or lesioning of the prefrontal cortex has been reported to alter heart rate, blood pressure, temperature, piloerection and respiration [1, 7, 9, 16, 22].

It has been established that fear-inducing stimuli activate the mesolimbocortical dopaminergic system, including the afferent dopamine (DA) projections of the MPFC [10, 70]. Predatory odours, social defeat stress, inescapable footshock, and systemic administration of anxiogenic drugs, all increase extracellular dopamine levels and/or turnover in the MPFC [3, 24, 49,

67, 71]. Furthermore, these increases in MPFC dopamine levels can be blocked by anxiolytics such as benzodiazepines [37, 68]. Moreover, the MPFC has direct and/or indirect connections to subcortical structures believed to be involved in fear and anxiety such as the amygdala, hippocampus, nucleus accumbens, caudate, hypothalamus, and the periaqueductal grey [28, 33, 42, 62, 66]. In addition, Fos-like immunoreactivity (Fos-LI), an indicator of functional activation, is expressed in the MPFC after exposure to a variety of anxiety-provoking challenges [12, 34, 47], and can be blocked by benzodiazepine anxiolytics [48]. Finally, human imaging studies have shown that activation of the MPFC is altered in normal subjects during exposure to fearful stimuli, and abnormal MPFC activity has been associated with a number of anxiety disorders (For a review see [41]).

Despite the evidence of MPFC involvement in the neural and hormonal correlates of fear and anxiety, studies examining the behavioural effects of MPFC lesions have been inconsistent and sometimes contradictory in their findings. In humans and primates, the effects of lesions of the MPFC on fear and anxiety are complex, with both increases and decreases of emotional responses being reported by investigators [17]. In the rat, behavioural studies have largely relied on conditioned fear responses to investigate the effects of MPFC lesions. In the conditioned fear test, electrolytic lesions of the ventral MPFC have been reported not to affect conditioned or unconditioned freezing, while retarding extinction [46]. Quirk and colleagues [56] reported no effects on extinction or acquisition, instead showing increased recovery of extinguished

conditioning. In contrast, dorsal MPFC lesions have been reported to increase freezing during all stages of conditioned fear testing in mice and in rats, suggesting a general increase in fear reactivity [45, 77]. Excitotoxic lesions of the MPFC have been shown to increase freezing to contextual cues, decrease freezing to the CS cue, but not affect extinction [36]. Interestingly, a study of the fear-potentiated startle response reported that MPFC lesions did not affect expression, acquisition or extinction [19]. Other studies investigating the effects of lesions on other aversive conditioning tasks have indicated impairment [15, 31, 40], enhancement [32], or no effect [26, 29, 35] on performance.

This inconsistent pattern of findings from MPFC lesion studies on conditioned fear is further complicated by the possible role of the MPFC in mnemonic processes. Evidence suggests that the prefrontal cortex may be involved in a variety on memory processes, such as working memory, reward, appetitive and even spatial learning [17, 60, 78, 79, 80]. Given the potential mnemonic functions of the region, it is possible that the changes in conditioned fear responses after lesioning are a result of memory dysfunction. Therefore, test examining unconditioned fear behaviour may provide advantageous assays of fear and anxiety, since they are not as susceptible to confounds such as the effects of lesions on learning and memory [58, 72]. However, studies examining the effects of MPFC lesions on unconditioned fear responses, although promising, have not been entirely uniform. MPFC lesions on open field behaviour have been reported to increase [26, 27, 31] and decrease [35, 36] fear responsivity as assessed by open field behaviour. Most studies investigating the

effects of MPFC lesions on elevated plus-maze behaviour show anxiolytic-like effects [20, 36, 40, 64], although there are exceptions [31, 35]. Other studies examining the effects of MPFC transections on the social interaction test, and MPFC lesions on food neophobia, have also reported anxiolytic-like effects [6, 20].

In addition to inconsistent findings, the majority of studies examining the effects of MPFC lesions on fear behaviour are limited by the use of non-excitotoxic lesioning methods. Since the MPFC has a large number of cortical and subcortical connections [8, 62], many fibers of passage course through the region and are destroyed by electrolytic, transection, or aspiration lesions. Therefore, these lesioning techniques may not be appropriate for studying the function of the MPFC, since it is not clear whether the effects of lesions are due specifically to destruction of MPFC neurons or to a loss of axonal connecting fibers.

Given the possible confounding effects of learning and memory, inconsistent findings, and questionable lesioning methodology of previous studies, more systematic investigation is needed to determine the effects of MPFC lesions on fear and anxiety. The purpose of the present study was to examine the effects of excitotoxic lesions of the MPFC on three behavioural assays of fear and anxiety: the elevated plus-maze, social interaction, and shock probe burying tests. All three tests are known to be sensitive in detecting the anxiolytic and anxiogenic profiles of a variety of clinically effective compounds, and have undergone behavioural and physiological validation (For a review see

[73]). The three tests utilize different fear-provoking stimuli and measure distinct fear responses, and therefore may provide robust convergent behavioural evidence for the putative role of the MPFC in mediating fear and anxiety.

2. Materials and Method

2.1. Subjects

The subjects were naïve, male Sprague-Dawley rats, purchased from *Ellerslie Research, University of Alberta, Canada*. Rats weighed between 350-450 grams at the time of surgery, and were individually housed in polycarbonate cages (47 x 25 x 20.5 cm) for the duration of the experiment. They were maintained on a 12:12 h light/dark cycle, with lights on at 0700 h, and were provided with food and water ad libitum. The treatment of all animals was in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Surgery

The subjects were administered an injection of atropine sulfate (0.1mg/0.2ml ip), to reduce respiratory complications, and were then anaesthetized with pentobarbital (Nembutal 50 mg/kg ip). Subjects were also hydrated with 0.9% saline (3 cc, ip) and treated with penicillin (Crystiben, Rhone Merieux, Canada, 15,000 IU/0.05 cc im). They were then placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA), an incision was made to

expose the skull, and small holes were drilled in the skull above the injection sites. Six intracranial infusions were administered at three bilateral sites (±0.7 mm ML for all following coordinates: +3.9 mm AP and –3.0 DV; +3.0 AP and –3.7 DV; +3.0 AP and –2.7 DV). Ibotenic acid (Sigma-Aldrich, Oakville, Ontario, 5 μg/0.5 μl) dissolved in phosphate-buffered saline (PBS), or the PBS alone, was infused through a 28 gauge needle attached by polyethylene tubing to a 10 μl Hamilton syringe. All infusions were delivered by an infusion pump (Harvard Apparatus 22), at a rate of 0.2 μl/min. The needle was left in place 3 minutes after the infusion to allow for diffusion. All holes drilled into the skulls of rats were covered with bonewax (Ethicon, Somerville, NJ). Immediately after surgery rats were given an injection of 0.9% saline (3 ml, ip) and were placed into a heated environment until they regained consciousness. Rats were then allowed to recover in their home cages before the start of behavioural testing.

2.3. Behavioural Testing

All behavioural testing was recorded on videotape. Behavioural testing commenced 15 days after surgery. On the days of testing rats were moved from their vivarium to a holding room, separate from the behavioural testing rooms, and were allowed to habituate to the holding room for at least 30 minutes prior to testing.

2.3.1. Handling and Resistance to Capture (RTC)

All rats were handled 4 consecutive days immediately prior to the first day of behavioural testing. Rats were handled gently, in their vivarium room, for 3 minutes on each day in order to habituate the animals to manipulation by the experimenter. On each of the 4 days a rating was given based on the reactivity of the rat to initial handling. A score on a 0-4 scale was assigned (0= no reaction 1= flinch movement or freezing, 2= movement away from experimenter, 3= running away from experimenter, 4= violent struggle and/or biting).

2.3.2. Elevated Plus-Maze Test

The maze was a plus-shaped apparatus, constructed from wood, and elevated at a height of 50 cm. It consisted of two 50 x 10 cm open arms, and two 50 x 10 x 50 cm enclosed arms, all arms having an open roof. Testing was conducted in a quiet room that was dimly illuminated (< 15 lux as measured on the surface of the plus-maze). All behavioural data were collected by a "blind" observer who was seated inside the testing room. All testing occurred between 1000-1600 h and behaviour was recorded for 5 minutes. Four variables were measured during each 5 minute test: 1) Time spent in the open arms. 2) Time spent in the closed arms. 3) Number of entries into the open arms. 4) Number of entries into the closed arms. A rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm. Time spent in the open arm was quantified as a percentage of total arm activity, i.e. the amount of time that the rat spent in the open arms relative to the total amount of time spent in all of the arms (open/total time). Entries into the open arms was similarly

quantified as a percentage of total arm entries (open/total entries) [52, 53]. A higher percentage of open arm time or open-arm entries were taken as measures of anxiolytic-like behaviour [52, 53]. In addition, the total number of closed arm entries, as well as the total number of all arm entries, were used as indexes of general activity [53, 59].

2.3.3. Social Interaction Test

The first of two social interaction tests was conducted four days after the final day of elevated-plus maze testing. The social interaction testing apparatus was a black Plexiglas box 60 X 60 X 40 cm. The testing arena was divided into nine 20 cm^2 squares by lines of yellow tape. All testing occurred between 1000-1600 h. In the first social interaction test rats were not habituated to the apparatus and were tested under bright lighting conditions (approximately 600 lux). All rats were paired with an unfamiliar conspecific of approximately the same weight (< 30 g difference) in the same treatment condition (i.e. lesion-lesion, sham-sham). A small mark made with a marking pen on the tail of one of the rats was used to distinguish the animals. Individual tests were 10 minutes in length. All behaviour was scored after testing by a "blind" observer. The duration of the following behaviours were recorded as social interaction when actively executed by the rat being scored: 1) sniffing of conspecific 2) following of conspecific 3) crawling under or over conspecific 4) grooming 5) wrestling 6) kicking or boxing 7) biting. All measures were combined to provide a total duration of social interaction. A longer duration of total social interaction is

believed to represent lower levels of fear and anxiety [14]. As a measure of general activity, the total number of line crossings was also recorded. Following completion of the first test, rats were returned to their home cages. The following day the rats were habituated to the testing apparatus under dim lighting (approximately 20 lux) conditions. The second social interaction test occurred two days after initial testing and followed the same procedure with the exception that testing occurred in a now familiar apparatus under dim lighting conditions. Rats were again tested with an unfamiliar conspecific in the same treatment group. The high lighting and the unfamiliarity of the apparatus in the first social interaction test have been shown to be anxiety-provoking, and testing under these conditions is known to be more sensitive in detecting increases in social interaction (i.e. anxiolytic-like effects) [14]. In contrast, since the duration of social interaction is higher in control animals under the conditions used in the second social interaction tests (i.e. dim lighting and familiarity with apparatus), the second version of the test is more sensitive in detecting decreases in social interaction, or anxiogenic-like effects [14].

2.3.4. Shock-Probe Burying Test

Four days after the final social interaction test, anxiety was assessed in rats using the shock-probe test. The shock probe apparatus consisted of a 40 X 30 X 40 cm Plexiglas chamber, evenly covered with woodchip bedding material. The shock probe was attached to one side of the chamber, 2 cm above the bedding. The shock probe was 6.5 cm long and 0.5 cm in diameter, and was

helically wrapped with two copper wires through which an electric current was administered. The electrical current was generated from a 2-pole regulated AC shocker (H13-01 2-pole shocker, Colbourn Instruments, Allentown, PA, USA). Rats were habituated individually in an identical Plexiglas chamber without the shock probe for approximately 40 minutes daily on the 4 consecutive days prior to shock-probe testing. All testing occurred between 1000-1800 h. On the test day, rats were individually placed in a corner of the chamber facing away from the shock–probe. The probe was electrified and rats coming into contact with the probe with their snout or forepaws received a 2 mA shock. The 15 minute observation period began once the first shock was delivered and the probe remained electrified throughout the entire testing period. The "blind" observer measured 1) Total amount of time spent spraying bedding material towards and on top of the shock probe with rapid alternating movement of the forepaws (i.e. burying behaviour), 2) Number of shocks received due to contact with the probe, 3) Amount of time spent immobile, as a measure of activity levels, and, 4) Reactivity to shock, which was measured on a 4 point scale (1= flinch involving head or paw, 2= whole body flinch with or without slow ambulation away from probe, 3= whole body flinch or jumping followed by immediate ambulation away from probe, 4= whole body flinch or jumping, accompanied by the rat running to the opposite end of the chamber) (54). A mean shock reactivity score was calculated and used in analysis. Duration of burying was the primary measure of fear and anxiety, with decreases in the durations of probe-burying representing anxiolytic-like effects [55]. All observations were made by a

"blind" observer who examined the outcome of testing via a video camera link in a separate room.

2.3.5. Shock-Probe Avoidance Retention

Eight days after initial shock-probe testing, the following retention test was conducted. Rats were tested in the same apparatus under identical conditions. Latency to contact the shock probe was measured and used as an index of retention of shock probe avoidance learning [39]. Contact of the probe was operationally defined as contact sufficient for an electric shock to be delivered.

2.4. Histology

Following the completion of behavioural testing rats were euthanized with an overdose of chloral hydrate and perfused intracardially with 0.9% (wt/vol) saline followed by an infusion of 4% (vol/vol) formaldehyde. Subjects' brains were then removed from the skull and placed in a postfix solution of 4% formaldehyde for a minimum of 48 hours. The brains were frozen with dry ice and then sectioned using a sliding microtome into 60 μm coronal sections. Every second section was collected. Brain sections were then mounted onto glass slides and stained with thionin. The brain sections were then examined under a microscope to determine the extent and location of the ibotenate-induced lesions.

2.5. Statistical Analyses

All results were expressed as means and standard errors of the mean (S.E.M.). Behavioural measures from the three anxiety tests were analyzed with analysis of variance (ANOVA) (α=.05). Handling scores were analyzed by an analysis of variance with days of handling and lesion condition as independent factors (Two-way ANOVA). Post-hoc significance testing was conducted with a least significant difference (LSD) test (α=.05). The test results from one rat were excluded due to excessive noise during testing.

3. Results

3.1. Histology

The ibotenic acid infusions produced lesions of reasonably uniform size and comparable volume. All subjects included in the analyses had extensive lesions of the prelimbic, infralimbic, and anterior cingulate areas, as well as variable damage to the frontal area 2, the dorsal peduncular, medial orbital and ventral orbital regions. *Figure 2-1* shows a diagram of the minimal and maximal extent of the ibotenic acid lesions. *Figure 2-2* is a photograph of a representative lesion. Data from three rats were excluded from the study due to excessive damage to the nucleus accumbens and/or an excessively asymmetrical pattern of damage.

3.2. Handling and RTC

Figure 2-3 displays the resistance to capture (RTC) scores from the first four days of handling. A two-way ANOVA (day X lesion) showed there was no main effect for RTC scores by group *(P=0.84)*, but a significant main effect by day *[F(3, 140)=26.65, P <0.001]*, and a significant group by day interaction *[F(3, 140)=2.97, P = 0.03]*. Post-hoc LSD testing of rats across days revealed a significant decrease in RTC scores from the first day of handling, on the second (*P=0.001*), third *(P<0.001)*, and the final day of handling *(P<0.001)*, strongly suggesting handling was effective in habituating rats to experimenter manipulation.

3.3. Elevated-Plus Maze Test

Figure 2-4 depicts the percentage of open-arms entries. Rats with MPFC lesions displayed a significantly greater percentage of open arm entries than controls *[F (1, 34)=6.66 , P=0.014]* suggesting an anxiolytic-like effect. *Figure 2-5* depicts the percentage of open-arm time. Rats with MPFC lesions displayed a significantly greater percentage of open-arm time than controls *[F (1, 34)=6.91 , P=0.013]* which also suggests an anxiolytic-like effect. There was no indication of changes in general activity level, since there was no significant effect between the groups in the total number of arms entered *[F (1, 34)=1.06 , P=0.31; Table 2-1]* or any significant differences in the number of closed arms entered *[F (1,34)=0.01, P=0.93; Table 2-1]*.

3.4. Social Interaction Test

Figure 2-6 depicts the total time spent in active social interaction during the first test and shows an anxiolytic-like effect of MPFC lesions. Rats with MPFC lesion displayed a significantly greater amount of time in social interaction than controls *[F (1, 33)=5.92, P=0.021]*. General activity did not appear to be affected by the lesion, as there was no significant differences between the groups on number of line crossings during the test *[F (1, 33)=1.89 , P=0.18; Table 2-1]*. The second social interaction test *(Figure 2-7)* did not reveal any significant differences between MPFC lesion and control rats in the duration of social interaction *[F (1, 33)=0.96, P=0.34]*.

3.5. Shock-Probe Burying Test

Figure 2-8 shows the total duration of time spent burying during the shock probe test. Lesioned rats spent significantly less time burying the shock probe than control rats *[F (1, 35)=5.57 , P=0.024]*, indicating an anxiolytic-like effect. The number of shocks taken during the 15 minute test was not affected by lesion *[F (1, 35)=0.018 , P=0.894, Table 2-1]*. The anxiolytic-like effect in the shock probe test occurred without any significant effects on shock reactivity *[F (1, 35)=0.099 , P=0.76; Table 2-1]* or immobility *[F (1, 35)=1.55 , P=0.22; Table 2-1]*.

3.6. Shock Probe Avoidance Retention

The latency to contact the shock probe during retention was not different between lesion and control animals *[F (1, 35)=0.72 , P=0.40, Table 2-1]*. These

results indicate that retention of shock-probe avoidance learning was unaffected by the pre-training lesions.

4. Discussion

The present study provides clear evidence that excitotoxic lesions of the rat medial prefrontal cortex (MPFC) attenuate fear-related responses in the elevated-plus maze, social interaction and shock probe burying tests. In the elevated plus-maze, it was found that rats with complete lesions of the medial prefrontal region displayed a significantly greater percentage of open arm entries and percentage of time spent in the open arms *(Figure 2-4, Figure 2-5)*, an anxiolytic-like effect suggesting a reduction in fear. In the social interaction test, lesioned rats spent a significantly longer period of time in active social interaction with a conspecific under anxiety-evoking conditions *(Figure 2-6)*, also suggesting an anxiolytic-like effect due to MPFC lesioning. Finally, in the shock probe burying test, MPFC lesioned rats spent significantly less time displaying defensive probe burying behaviour, providing further evidence that lesions of the MPFC have an anxiolytic-like effect *(Figure 2-8)*. Altogether, the findings of the present study provide strong evidence that lesions of the MPFC are capable of decreasing fear and anxiety-related behaviour in rats, and they suggest that the region has a significant role in a wide variety of fear and anxiety responses.

To our knowledge this is the first study to examine the effects of excitotoxic MPFC lesions on the social interaction and the shock probe burying tests, and in our estimation this is the first study to use three pharmacologically well-validated assays of fear and anxiety (For a review see [73]). Our finding of a decrease in fear responsiveness due to lesioning is strengthened by the fact that the anxiolysis was found across a broad range of fear responses: open arm avoidance, social inhibition and defensive burying. It is also important that all significant results were consistently anxiolytic-like (i.e. no anxiogenic-like effects were found). In particular, the second social interaction test, which was specifically designed to be sensitive in detecting anxiogenic-like effects (and correspondingly less likely to detection anxiolytic-like effects), was not significant, and in fact showed a trend towards an anxiolytic-like effect *(Figure 2-7)*. Therefore, the findings of this study give unambiguous and robust support to the notion that MPFC lesions attenuate fear behaviour.

In addition, several potential confounds were shown not to contribute to the results of the present study. All three tests included measures of general activity levels, and no significant differences were found between lesioned and sham-lesioned animals on any of these measures. In addition, while an anxiolytic profile in the elevated plus-maze and the social interaction tests entails an *increase* in activity (i.e. open arm exploration and active social behaviour) anxiolytic-like effects in the shock-probe burying test involve a *decrease* in activity (i.e. burying behaviour). Therefore, the anxiolytic-like effects in the

three tests cannot be explained as arising from a simple change in general activity, general arousal, or as a simple disinhibition of motor responses.

While the elevated plus-maze and social interaction tests do not involve an explicit learning or memory requirement, it could be argued that the shock-probe test does require that the rat form and retain an association between the shock and the probe. However, shock probe avoidance learning and memory was not affected by MPFC lesion during acquisition (number of shocks) or retention (latency to contact probe) testing *(Table 2-1)*. Therefore, the decrease in probe burying was not simply a result of a deficit in the ability to associate the probe with the electric shock. In addition, pain sensitivity, as measured by shock reactivity during shock probe testing, was also not affected by MPFC lesions *(Table 2-1)*. Taken together, these results suggest that the anxiolytic-like effects of MPFC lesions in the present study were not confounded by changes in memory, general activity, or pain sensitivity. It is therefore reasonable to conclude that the findings of this study are a result of a *specific* attenuation of fear and anxiety-related behaviour.

While the present study and the majority of previous studies examining the effects of MPFC lesions on the elevated plus-maze are consistent in finding anxiolytic-like effects [20, 36, 40, 64], it is not entirely clear why two MPFC lesion studies have found either anxiogenic-like effects [31] or no effects [35] in the elevated plus-maze. One possible explanation for the latter finding may be the methodological differences that exist between different laboratories (For a review see [25]). A wide variety of testing conditions, such as handling of

animals, ambient lighting, time of day, maze construction, and prior maze exposure, have been shown to alter performance on the elevated plus-maze [4, 13, 21, 59]. In particular, handling regimen has been noted to have crucial effects on plus-maze behaviour [25]. Interestingly, resistance-to-capture (RTC) scores were shown to decline across the days of handling *(Figure 2-3),* and an interaction between lesioning and day of handling was found. These results emphasize the importance of handling in the habituation of animals after MPFC lesioning, and, suggest it may be necessary to decrease stress-related effects resulting from the invasive surgical procedure used. Jink and McGregor [31], who found anxiogenic-like effects in the elevated plus-maze after electrolytic lesioning of the MPFC, reported that handling was minimized prior to behavioural testing. This may in part explain the anxiogenic-like effect reported by the study, which is contrary to the findings of most lesion studies. Another study by Lacroix and colleagues [35] reported no significant effects of lesions on the elevated plus-maze. In this study, the extent of the lesion may have been a crucial factor in their non-significant result. The lesions were primarily located on the dorsal MPFC, with large portions of the infralimbic cortex often left intact. It is therefore possible that the lesions induced in this study were not extensive enough to affect plus-maze performance.

We know of only one previous study that examined the effects of MPFC lesions in the social interaction test. Gonzalez and colleagues [20] reported that medial prefrontal *transections* enhance social interaction. The findings of the present study are consistent with the Gonzalez study, but also demonstrate that

discrete, axon-sparing excitotoxic lesions of the MPFC increase active social interaction. Similarly, only one other study has examined the effects of MPFC lesions in the shock probe burying test. This study reported that electroylytic MPFC lesions did not affect the burying response in the shock probe test of anxiety [40]. The simplest explanation for the lack of an effect in this study is that the lesions were relatively specific to the *prelimbic* region, rather than the entire MPFC. These experimenters did not extensively damage the infralimbic or anterior cingulate regions. Therefore, it might be possible that specific prelimbic lesions are not sufficient to decrease defensive burying. In addition, the finding may be a result of the unconventional behavioural methodology used by Maaswinkel and colleagues. The researchers habituated the rats immediately prior to administration of the shock probe test and tested animals in their housing room. The methodology typically employed in the pharmacologically-validated version of the test requires a 24h period between the last session of habituation and behavioural testing; in addition, testing is conducted in a separate room from which the animals are housed [74].

While the present study demonstrates that lesions of the whole medial prefrontal region lead to the attenuation of a variety of fear-related responses, it must be noted that functional and anatomical differences exist within the rat medial prefrontal cortex, particularly between the dorsal and ventral areas of the MPFC. The dorsal and ventral subregions of the medial prefrontal cortex show different patterns of connectivity with cortical and subcortical structures [2, 8, 30, 62]. Important cytoarchitectonic distinctions also exist, with the infralimbic

region appearing to be periallocortical, while other medial prefrontal areas show less primitive lamination patterns [81]. Therefore, anatomically there is reason to believe that the subregions of the MPFC differentially contribute to behavioural processes. Furthermore, it is known that the different subregions of the MPFC have unique functions in the processing of particular cognitive tasks. Differences have been found between subregions in strategy switching, foraging tasks, conditioned place preference and behavioural sensitization [57, 61, 75]. These findings suggest that regional differences within the MPFC in regard to the mediation of anxiety may also exist. Indeed, distinctions between the dorsal and ventral regions of the medial prefrontal cortex in the mediation of fear-related behaviour have been suggested. Morgan and Ledoux [45] reported that dorsal MPFC lesions appear to increase fear reactivity, while ventral MPFC lesions specifically impair extinction of a conditioned fear. Lesions centered on the dorsal MPFC have been reported to increase fear reactivity by other researchers [26, 27, 31], and have also been described as having no effect on different assays of anxiety [35, 40]. Most of these findings are complicated by the use of non-excitotoxic lesioning and conditioned fear tests to assess anxiety, but they do suggest that a degree of functional specialization, within the MPFC, over the processing of fear and anxiety may be present. Thus, these possible functional differences within the MPFC may also be an underlying cause of the inconsistent findings of previous research examining the role of the region in fear and anxiety, since there is a great deal of variability in lesion size and

placement between the studies. Clearly, more research into the specific effects of subregional manipulation on fear responses is required.

Intriguingly, the pattern of findings in the elevated plus-maze and the shock probe test after MPFC lesions in the present study resemble the effects of septal lesions previously found on these tasks. It has been shown that excitotoxic and electrolytic lesions of the medial and lateral septum increase open arm activity and decrease defensive burying, suggesting anxiolysis [43, 54]. Interestingly, Treit and Menard [44] found that electrolytic lesions of the dorsal hippocampus or amygdala produced selective deficits in shock-probe avoidance, without altering open arm activity or burying behaviour. The similarities in the effects of MPFC lesions and septal lesions suggest that these two regions may collaborate in the mediation of anxiety. Since the MPFC has been shown to have connections with the medial septum [18], a functional connection between the regions in relation to fear and anxiety is an intriguing possibility. Further research is required to establish the precise relationship between the MPFC and the septal region in regards to fear and anxiety.

It has been argued that the prefrontal cortex does not have a primary role in mediating basic fear and anxiety responses, but rather acts as an inhibitory center of control by which neocortical regions modulate fear responses, based on complex temporal and/or contextual information processed by the cortex [38]. This notion seems to suggest that the prefrontal region has a secondary function in terms of the neural systems that direct fear-related behaviour. However, since the findings of the present study revealed a consistent attenuation of fear

reactivity in a very wide range of fear behaviours, they suggest that the region may have a more direct role in mediating fear behaviour. The breadth of the findings suggests that the MPFC region has a general role in fear processing; in other words, the MPFC may be a center of co-ordination of the basic behavioural responses related to fear and anxiety. There is evidence to support this notion. For example, it is known that the region has direct connections with crucial diencephalic, midbrain and brainstem structures which likely direct simple fear-related physiology and behaviour, such as the periaqueductal gray, hypothalamus, solitary nucleus, dorsal raphe, and ventral tegmental area [28, 51, 62, 69] (For a review see [76]). In addition, stimulation or disruption of the prefrontal cortex also alters the basic physiological correlates of anxiety, such as respiration, heart rate, piloerection, blood pressure [1, 7, 9, 16, 22]. It may be the case that the MPFC exerts control over these regions directly without limbic structures, such as the amygdala, acting as intermediaries. Therefore, it is possible that the MPFC has a direct and active role in the neural mediation of fear behaviour. Further investigation is required to clarify the respective roles of the MPFC and other brain structures, which are known to be involved in emotional processing, in the neural mediation of fear and anxiety.

In summary, the present study provides clear evidence that lesions of the medial prefrontal cortex attenuate fear and anxiety-related behaviour. The significant and complementary findings from the elevated plus-maze, social interaction, and shock probe burying tests, which together examine a robust diversity of fear behaviour, greatly enhance the findings. In general, the results

of this study strongly suggest the medial prefrontal cortex is an important component of the neural architecture mediating fundamental fear-related behaviours.

Figure 2-1. Schematic diagram of coronal rat brain sections illustrating the maximal (coloured gray) and minimal (coloured black) area of damage induced by ibotenic acid lesioning. Cg1= Anterior cingulate area; DP= Dorsal peduncular area; IL= Infralimbic area; PrL= Prelimbic area; M2= Frontal Cortex, Area 2; MO= Medial orbital area; VO= Ventral orbital area. The atlas plates are adapted from Paxinos and Watson (1986).

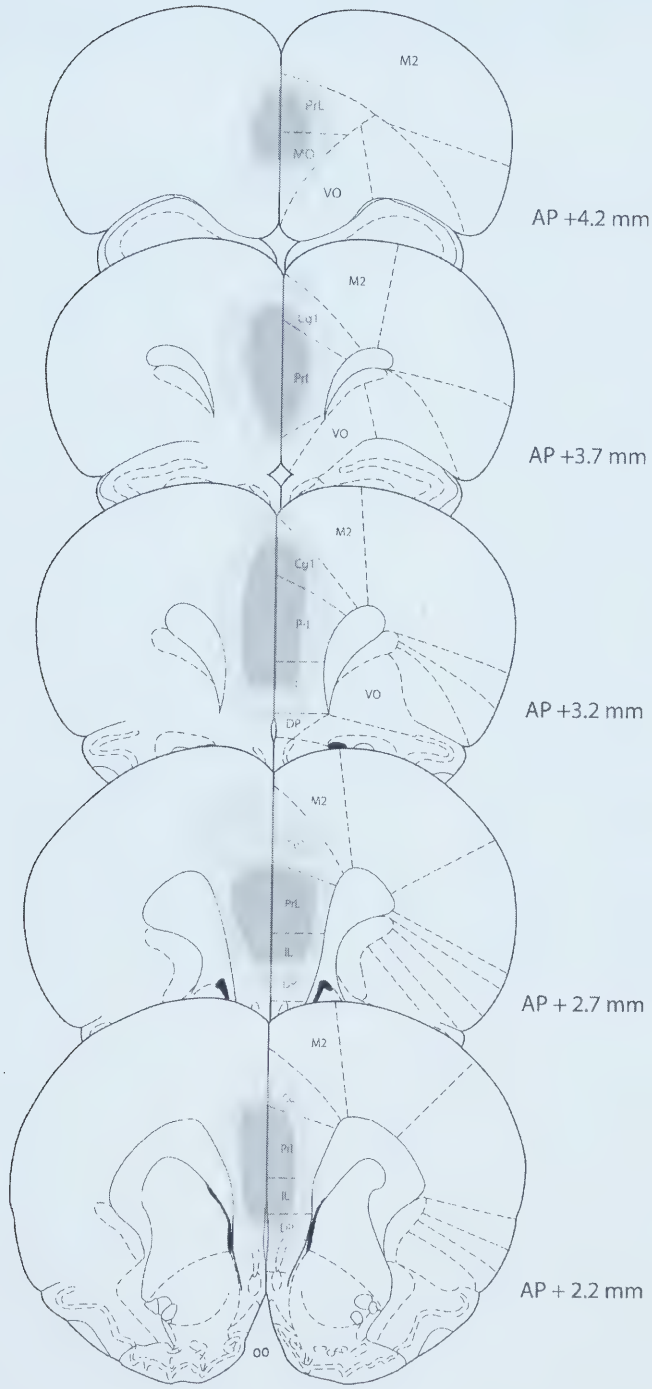
M2
PrL
VO
AP +4.2 mm
M2
Cg1
Prl
VO
AP +3.7 mm
M2
Cg1
P-l
VO
DP
AP +3.2 mm
M2
PrL
IL
AP + 2.7 mm
M2
Prl
IL
AP + 2.2 mm

Figure 2-2. Photomicrographs of thionin-stained coronal sections of the medial prefrontal cortex region. Sham lesioned rat brain (Left), Ibotenic acid lesioned rat brain (Right). Length of calibration bar is equal to 200 μm.

Figure 2-3. Mean (S.E.M.) resistance to capture (RTC) scores for sham and lesioned rats, on the four initial days of handling. ** P< .01 compared with RTC scores of rats on the first day of handling.

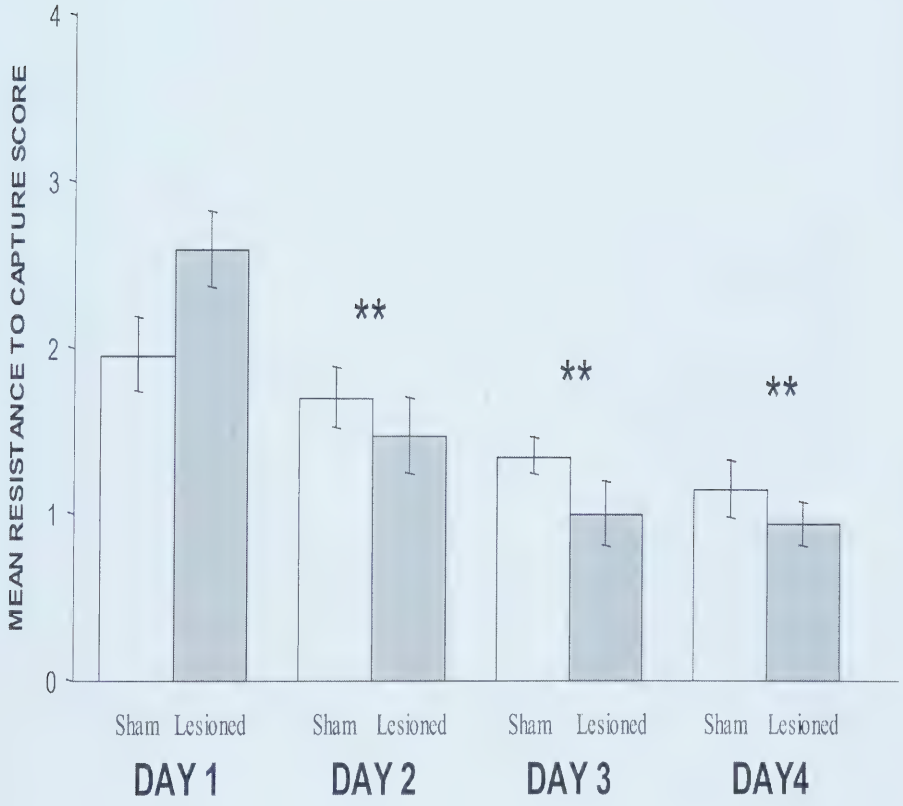
MEAN RESISTANCE TO CAPTURE SCORE
4
3
2
1
0
**
**
**
Sham Lesioned
Sham Lesioned
Sham Lesioned
Sham Lesioned
DAY 1
DAY 2
DAY 3
DAY4

Figure 2-4. Mean (S.E.M.) percent open arm entries in the elevated plus-maze. Sham operated versus medial prefrontal cortex lesioned rats. * P< .05 compared with the sham operated group.

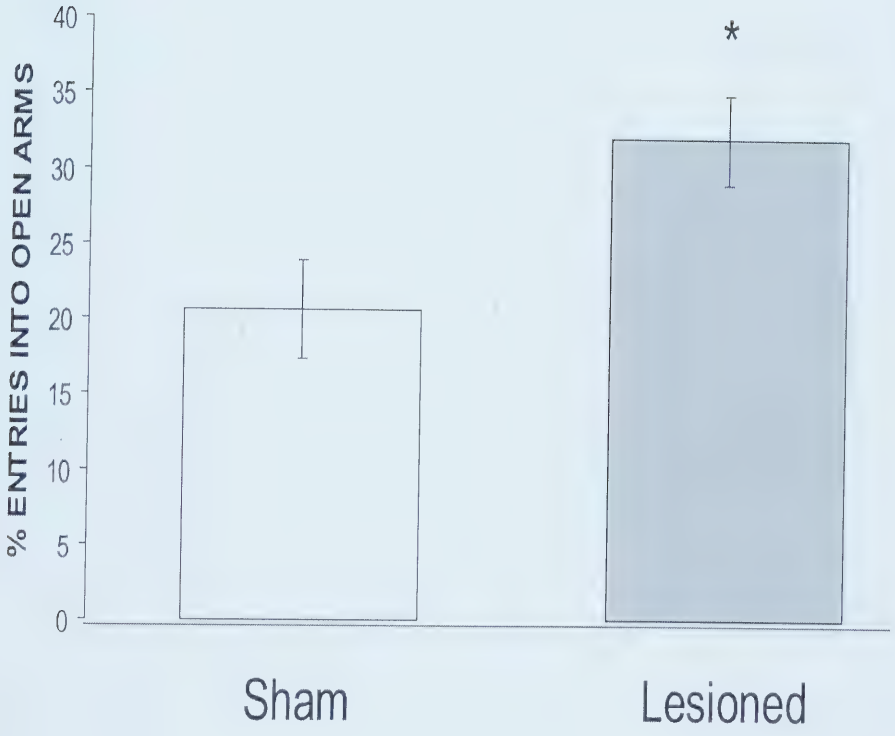
% ENTRIES INTO OPEN ARMS
40
35
30
25
20
15
10
5
0
*
Sham
Lesioned

Figure 2-5. Mean (S.E.M.) percent open arm time in the elevated plus-maze. Sham operated versus medial prefrontal cortex lesioned rats. * $P < .05$ compared with the sham operated group.

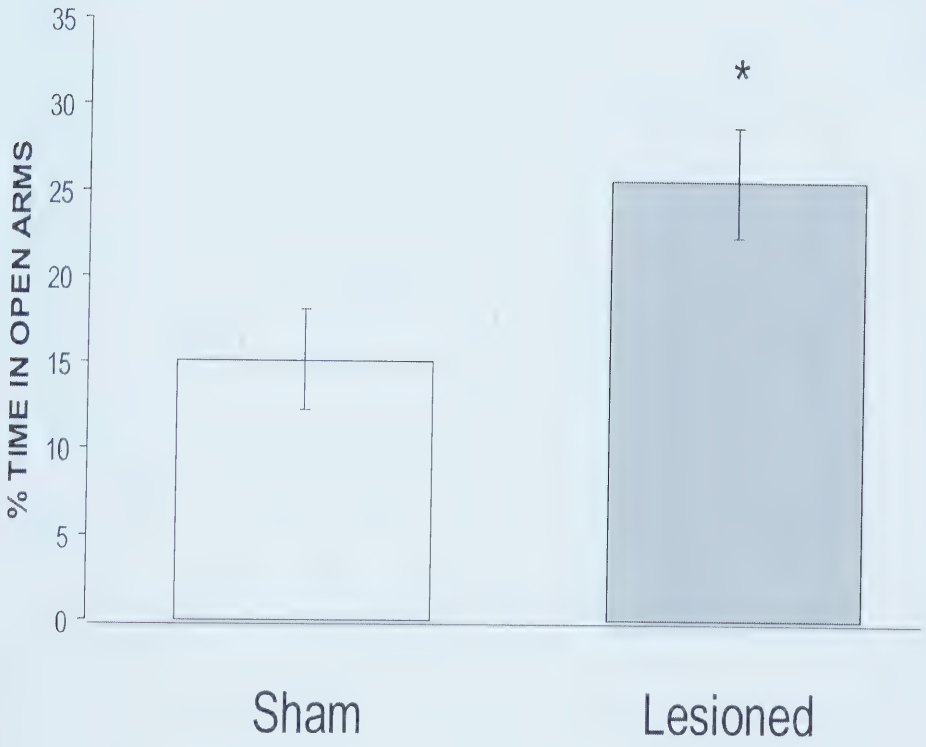
% TIME IN OPEN ARMS
35
30
25
20
15
10
5
0
*
Sham
Lesioned

Figure 2-6. Mean (S.E.M.) total duration of social interaction (seconds) in the first social interaction test. Sham operated versus medial prefrontal cortex lesioned rats. A line break occurs in the Y-axis between 30-95 seconds. * P< .05 compared with the sham operated group.

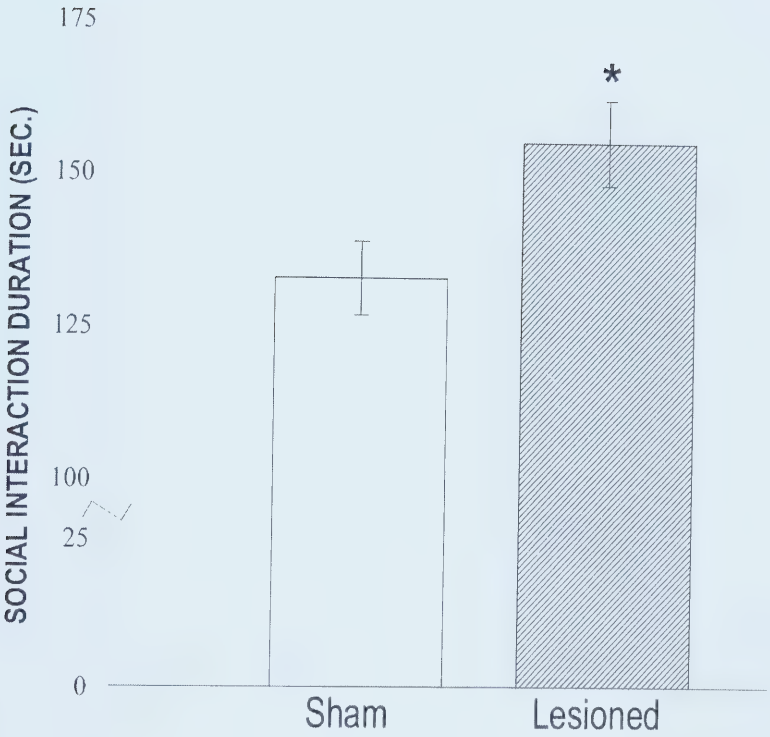
SOCIAL INTERACTION DURATION (SEC.)
175
150
125
100
25
0
*
Sham
Lesioned

Figure 2-7. Mean (S.E.M.) total duration of social interaction (seconds) in the second social interaction test. Sham operated versus medial prefrontal cortex lesioned rats. A line break occurs in the Y-axis between 30-95 seconds.

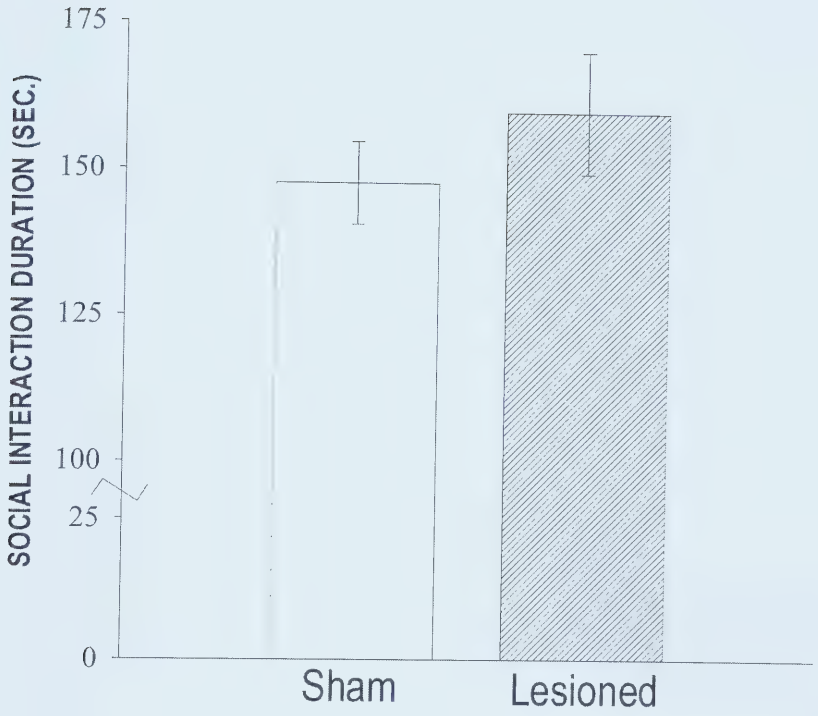
SOCIAL INTERACTION DURATION (SEC.)
175
150
125
100
25
0
Sham
Lesioned

Figure 2-8. Mean (S.E.M.) total duration of burying during the shock probe test. Sham operated versus medial prefrontal lesioned animals. * $P < .05$ compared with the sham operated group.

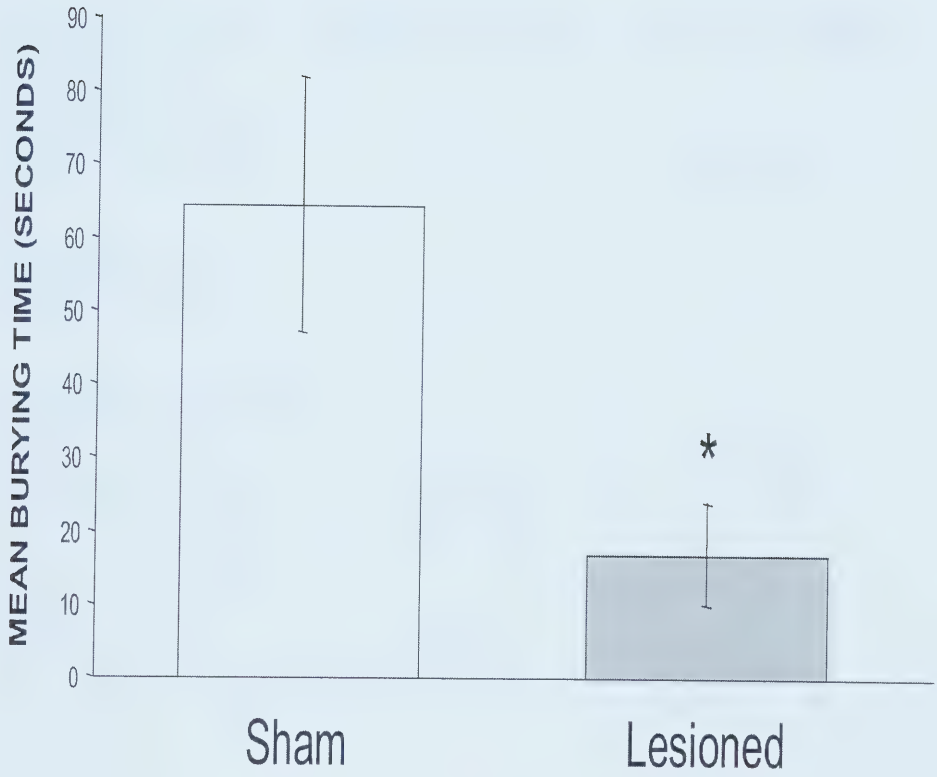
MEAN BURYING TIME (SECONDS)
90
80
70
60
50
40
30
20
10
0
*
Sham
Lesioned

Table 2-1. Table entries show the mean (±S.E.M.) total and closed arm entries in the elevated plus-maze, and number of line crossings in the social interaction test. In addition, the table shows the mean (± S.E.M.) number of shocks taken, duration of immobility, and shock reactivity during the shock-probe test, and latency to contact the probe during shock probe retention testing.

	SHAM OPERATED	MPFC LESIONED
Elevated Plus-Maze		
Number of subjects	19	17
Number of all arm entries	11.32 ± 1.03	12.71 ± 0.85
Number of closed arm entries	8.74 ± 0.72	8.82 ± 0.72
Social Interaction Test		
Number of subjects	18	17
Number of line crossings	113.44 ± 9.49	130.88 ± 8.33
Shock-Probe Burying Test		
Number of subjects	20	17
Number of shocks taken	2.2 ± 0.16	2.24 ± 0.22
Mean shock reactivity	2.0 ± 0.10	2.04 ± 0.08
Duration of immobility	3.9 ± 2.18	10.47 ± 5.12
Shock-Probe Retention		
Number of subjects	20	17
Latency to contact probe	480.65 ± 74.68	575.65 ± 83.51

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Chapter 3

Infusions of Midazolam into the Medial Prefrontal Cortex Produce Anxiolytic Effects in the Elevated Plus-Maze and Shock-Probe Burying Tests

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1. Introduction

The medial prefrontal cortex (MPFC) has been implicated in many fear and anxiety-related processes. For example, in rats the MPFC has been shown to be involved in physiological and endocrine correlates of fear and stress such as changes in respiration, HPA axis responses, heart rate, and blood pressure [1, 8, 10, 16, 56]. Specific neurochemical responses within the MPFC also seem to be correlated with fear and stress. For example, predatory odours, social defeat stress, inescapable foot-shock, and systemic administration of anxiogenic drugs, all consistently increase extracellular dopamine levels or turnover in the MPFC [3, 15, 23, 20, 40, 41, 59, 61]. In addition, increases in norepinephrine and serotonin levels or turnover have also been shown to result from exposure to fear-provoking stimuli [15, 20, 22, 41, 70]. Similarly, it has been shown that Fos-like immunoreactivity (Fos-LI), seen as an indicator of functional activation, is also increased in the MPFC after exposure to fear-provoking stimuli [12, 39]. Additionally, the MPFC has connections to a number of structures believed to be important in mediating fear and anxiety such as the amygdala, septum, hippocampus, hypothalamus, and the periaqueductal grey [27, 32, 53, 58].

Several studies have examined the effects of MPFC lesions on fear and anxiety as assessed by a wide variety of behavioural assays including conditioned freezing, elevated plus-maze, open field, shock-probe burying, and social interaction [19, 25, 26, 30, 33, 38, 54, 55]. Although these studies have

not always been uniform in the direction of their findings, some of the most recent studies have shown that MPFC lesions produce a reliable pattern of fear-reduction in more than one behavioural assay of fear and anxiety [19, 33, 54].

Benzodiazepines are widely known to be highly effective anxiolytics and are frequently used in the treatment of anxiety disorders [14]. It is known that benzodiazepine receptors are densely represented throughout the prefrontal cortex in rats [71]. A number of studies have shown that peripheral administration of benzodiazepine anxiolytics attenuate fear and/or stress-induced increases in extracellular levels or turnover of dopamine and norepinephrine [15, 31, 34, 48, 60, 69]. In addition, it has been shown that stress-induced Fos-LI is attenuated throughout the MPFC by the benzodiazepine lorazepam [39]. Other studies have shown that the $GABA_A$ agonist muscimol attenuates stress-induced dopamine and nitric oxide release when infused into the MPFC [11, 28], while intra-MPFC infusions of the $GABA_A$ antagonist picrotoxin increased MPFC dopamine release [51]. Interestingly, studies have also demonstrated that rats exposed to stressors show decreased levels of $GABA_A$ and benzodiazepine receptor binding in the frontal cortex [21, 24]. While these studies suggest that the $GABA_A$ /benzodiazepine receptor population of the MPFC may be involved in the neural processing of fear-related behaviour, to our knowledge no study has directly examined this by investigating the effects of intra-MPFC infusions of a GABAergic agonist or antagonist on fear-related behaviour.

While a diverse body of research has suggested that the MPFC is an important structure for the mediation of fear and anxiety, there is evidence to

suggest that the dorsal and ventral subregions of the MPFC may play unique roles in mediating fear-related behaviour. Lesions limited to the dorsal MPFC have been reported to increase fear reactivity [25, 26, 30, 37], while lesions extending to the ventral portion of the MPFC have generally produced an attenuation of fear-related behavioural responses [19, 33, 54]. In addition, ventral MPFC lesions have been shown to impair extinction of conditioned freezing, while dorsal lesions have been reported to have no effect on extinction [37, 38]. The dorsal and ventral MPFC also have anatomical differences, which may underlie the possible functional differences seen between the two regions. For instance, the dorsal and ventral subregions of the MPFC have different efferent and afferent patterns of connectivity with other brain structures [2, 9, 29, 53]. Furthermore, important cytoarchitectonic differences exist. In particular, the infralimbic region of the ventral MPFC shows periallocortical lamination, a more primitive lamination pattern than that of the dorsal MPFC [72]. Together, these findings suggest that the dorsal and ventral subregions of the MPFC may have different functions in the neural processing of fear and anxiety-related behaviour.

The purpose of the present study was to investigate the effects of intra-MPFC infusions of the benzodiazepine midazolam on the elevated plus-maze and the shock-probe burying tests. Both tests are well-validated models of fear and anxiety that are known to be effective in detecting the anxiolytic properties of a variety of compounds, including benzodiazepines [63]. Additionally, since previous research has suggested a possible functional difference between the

dorsal and ventral subregions of the MPFC on fear-related behaviour, the present study examined the effects of intracranial midazolam infusions into two sites: either the dorsal (anterior cingulate and dorsal prelimbic) or ventral (infralimbic and ventral prelimbic) MPFC.

2. Material and Methods

2.1. Subjects

The subjects were 56 male Sprague-Dawley rats, obtained from Ellerslie Research, University of Alberta, Canada. Rats were 58-71 days old and weighed 300-450 grams at the time of surgery. Rats were individually housed in polycarbonate cages (47 x 25 x 20.5 cm) for the duration of the experiment. They were maintained on a 12:12 h light/dark cycle, with lights on at 0700 h, and were provided with food and water ad libitum. The treatment of all animals was in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.2. Surgery

The subjects were administered an injection of atropine sulfate (0.1mg/0.2ml ip), to reduce respiratory complications, and were then anaesthetized with pentobarbital (Nembutal 50 mg/kg ip). Subjects were also hydrated with 0.9% saline (3 cc, ip) and treated with penicillin (Crystiben, Rhone Merieux, Canada, 15,000 IU/0.05 cc im). They were then placed in a stereotaxic

apparatus (Kopf Instruments, Tujunga, CA, USA), and an incision was made to expose the skull. Stainless steel 22-gauge guide cannulae (Plastics One, Roanoke, VA, USA) were bilaterally implanted targeting the dorsal or ventral medial prefrontal cortex (dorsal: +2.9 mm AP, ±1.9 ML, -0.9 DV; ventral: +2.9 AP, ±1.9 ML, -2.8 DV; all cannulae were angled at 15° towards the midline). The cannulae were secured to the skull with four jeweller's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula in order to keep the cannula tract clear. Immediately after surgery rats were given an injection of 0.9% saline (3 cc, ip) and were placed into a heated environment until they regained consciousness. Rats were then allowed to recover in their home cages before the start of behavioural testing.

2.3. Intracranial Infusion Procedure

Rats were randomly assigned to either the control condition or drug treatment condition prior to commencement of behavioural testing. Infusions were delivered via an infusion pump (Harvard Apparatus 22), at a rate of 0.5 µl/min. Midazolam (Maleate Salt; 5 µg/0.5 µl, Sigma-Aldrich, Oakville, Ontario) was dissolved in a phosphate-buffered saline (PBS). Rats were infused with midazolam (drug) or PBS alone (vehicle), through a 26 gauge stainless steel internal infusion cannula attached by polyethylene tubing to a 10 µl Hamilton syringe. The internal infusion cannulae extended 1.2 mm below the ventral tip of the implanted guide cannula, and bilateral infusions were conducted simultaneously. The internal infusion cannulae were left in place one minute

after the end of the infusion period to allow for diffusion. Midazolam was selected in preference to other benzodiazepines because of its superior water solubility, and, its widespread experimental and clinical use. The dose of midazolam was selected based on previous intracranial infusion studies reporting an anxiolytic effect (for a review see [36]).

2.4. Behavioural Testing

All behavioural testing was recorded on videotape. Behavioural testing occurred at least 7 days following surgery. All rats were handled by the experimenter for 3 minutes, and the cannulae tracts were checked for blockage, on the 4 consecutive days prior to the start of behavioural testing. On testing days, rats were moved from their vivarium to a holding room, separate from the behavioural testing rooms, and were allowed to habituate to the holding room for at least 30 minutes prior to testing. All testing commenced 3 minutes after the end of the intracranial infusion procedure. Rats were placed in the same treatment group (vehicle or drug) for both behavioural tests.

2.4.1. Elevated Plus-Maze Test

The maze was a plus-shaped apparatus, constructed from wood, and elevated at a height of 50 cm. It consisted of two 50 x 10 cm open arms, and two 50 x 10 x 50 cm enclosed arms, all arms having an open roof. Testing was conducted in a quiet room that was dimly illuminated (< 15 lux as measured on the surface of the plus-maze). All behavioural data were collected by a "blind"

observer who was seated inside the testing room. All testing occurred between 1000-1800 h and behaviour was recorded for 5 minutes. The maze was cleaned with distilled water after each rat was tested. Four variables were measured during each 5 minute test: 1) Time spent in the open arms. 2) Time spent in the closed arms. 3) Number of entries into the open arms. 4) Number of entries into the closed arms. A rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm. The time spent in the open arms was quantified as a percentage of total arm activity, i.e. the amount of time that the rat spent in the open arms relative to the total amount of time spent in all of the arms (open/total time x 100; [42, 43]). The number of open arm entries was similarly quantified as a percentage of the number of total arm entries (open/total entries x 100; [42, 43]). A higher percentage of open arm time or open-arm entries was taken as a measure of anxiolysis [43, 44]. In addition, the total number of closed arm entries, as well as the total number of all arm entries, were used as indexes of general activity [44, 50].

2.4.2. Shock-Probe Burying Test

One week after elevated plus-maze testing, fear-related behaviour was assessed with the shock-probe burying test. The shock probe apparatus consisted of a 40 X 30 X 40 cm Plexiglas chamber, evenly covered with woodchip bedding material, with an electrified probe protruding from one side of the chamber 2 cm above the bedding. The electrified probe, or 'shock-probe', was 6.5 cm long and 0.5 cm in diameter, and was helically wrapped with two copper

wires though which an electric current was administered. The electrical current was generated from an AC shocker (H13-15 precision regulated shocker, Colbourn Instruments, Allentown, PA, USA). Rats were habituated individually in an identical Plexiglas chamber without the shock probe for 45-60 minutes daily on the 4 consecutive days prior to shock-probe testing. All testing occurred between 1000-1800 h. At the start of shock-probe testing, rats were individually placed in a corner of the testing chamber facing away from the shock–probe. The probe was electrified and rats coming into contact with the probe with their snout or forepaws received a 2 mA shock. The 15 minute observation period began once the first shock was delivered and the probe remained electrified throughout the entire testing period. The following four behavioural measures were recorded: 1) Total amount of time spent spraying bedding material towards and on top of the shock probe with rapid alternating movement of the forepaws (i.e. burying behaviour), taken as a measure of fear-behaviour; 2) Number of shocks received due to contact with the probe, taken as a measure of aversive learning/memory; 3) Amount of time spent immobile (primarily consisting of rest and sleep), taken as a measure of general activity; 4) Reactivity to shock, which was measured on a 4 point scale (1= flinch involving head or paw, 2= whole body flinch with or without slow ambulation away from probe, 3= whole body flinch or jumping followed by immediate ambulation away from probe, 4= whole body flinch or jumping, accompanied by the rat running to the opposite end of the chamber) [45]. A mean shock reactivity score was calculated for each rat (i.e. the mean of the reactivity scores for all shocks received), and was

used as a measure of pain sensitivity. The duration of burying was used as the primary measure of fear-related behaviour, with decreases in the duration of probe burying indicative of an attenuation of fear and anxiety, i.e. anxiolysis [65]. All observations were made by a "blind" observer who examined the outcome of testing via a video camera link in a separate room. After each rat was tested any soiled bedding and all fecal boli were removed from the testing chamber. A layer of fresh bedding material was added and was smoothed to an even thickness of 3.75 cm.

2.5. Histology

Following the completion of behavioural testing rats were euthanized with an overdose of chloral hydrate and perfused intracardially with 0.9% (wt/vol) saline followed by an infusion of 4% (vol/vol) formaldehyde. Subjects' brains were then removed from the skull and placed in a postfix solution of 4% formaldehyde for a minimum of 48 hours. The brains were frozen with dry ice and then sectioned using a sliding microtome into 60 μm sections. Every second section was collected. Brain sections were then mounted onto glass slides and stained with thionin. The brain sections were then examined by a "blind" observer to determine the location of the cannulae implants. The behavioural data of rats with inappropriately placed cannulae (n= 19), or of rats showing substantial necrosis were excluded from the analysis (n= 2). Rats were deemed to have inappropriate placements if one or both of the cannulae infusion sites were located outside of the dorsal or ventral MPFC. This assessment was made

based on microscopic examination of the thionin stained brain sections. Six rats with dorsal MPFC cannulae implants and 6 with ventral implants were given intra-MPFC infusions (0.5 μl/min) of the Chicago blue stain (2.5% dissolved in PBS), 15 minutes prior to perfusion. Histological processing for these rats (perfusion, sectioning, thionin staining) was otherwise identical to the procedure described above. The Chicago blue stain was used to estimate the extent of diffusion of the intracranial infusions performed in the study.

2.6. Statistical Analyses

All results were expressed as means and standard errors of the mean (S.E.M.). Behavioural measures from the two anxiety tests were analyzed by analysis of variance with drug treatment (Midazolam or Vehicle alone) and infusion site (Dorsal or Ventral MPFC) as independent factors (two-way ANOVA; α=0.05). In addition, the correlation between the primary measures of anxiety-like behaviour (% time in open arms, % entries in open arms, time spent burying) and the dorsal/ventral location of the infusion sites was also assessed by calculating three separate Pearson product-moment correlation coefficients. Finally, two-sample t-tests were used to assess whether differences existed between vehicle and midazolam infused rats having one or both infusion sites outside of the MPFC, in order to determine whether drug effects were specific to the MPFC.

The test results from 2 rats were excluded from the analysis of the elevated plus-maze data due to excessive noise during testing. The test results

from 1 rat was excluded from the analysis of the shock-probe data because the subject did not receive a shock within 15 minutes of being placed in the shock-probe apparatus. In addition, 7 rats were only tested in the elevated plus-maze but not the shock-probe test because their acrylic 'head cap', which was used to secure the cannulae to the skull of the rat, became unstable or fell off during the period between the two behavioural tests.

3. Results

3.1. Histology

Fig. 3-1 shows the approximate infusion sites of the dorsal and ventral MPFC cannulae included in the overall analysis. Rats implanted with cannulae targeting the dorsal MPFC had infusion sites within the anterior cingulate or dorsal prelimbic regions of the MPFC, while rats with ventral MPFC cannulae implants had infusion sites in the infralimbic and ventral prelimbic regions. *Fig. 3-2* shows the area covered by diffusion of intracranial infusions aimed at the dorsal (black) and ventral (grey) MPFC for all rats given infusions of Chicago blue stain. This figure illustrates that dorsal MPFC infusions were primarily limited to the anterior cingulate and dorsal prelimbic subregions of the MPFC, while ventral MPFC infusions were primarily limited to the infralimbic and ventral prelimbic subregions of the MPFC.

3.2. Elevated Plus-Maze Test

Fig. 3-3 depicts the percentage of open arm entries. Rats receiving infusions of midazolam had a significantly higher percentage of open arm entries than controls ($F(1,50)=4.217$, $P=0.05$) . There was no significant difference between the scores of rats with dorsal or ventral implants ($F(1,50)=0.158$, $P=0.69$). Also, there was no significant interaction between the drug treatment (vehicle or midazolam) and the site of the infusion (dorsal or ventral) on the percentage of open arm entries ($F(1,50)=0.013$, $P=0.91$). Fig. 3b depicts the percentage of open arm time. Rats receiving infusions of midazolam spent a significantly greater percentage time in the open arms than controls ($F(1,50)=6.302$, $P=0.02$) . There was no significant difference between the scores of rats with dorsal or ventral implants ($F(1,50)=0.032$, $P=0.86$). Also, there was no significant interaction between the drug treatment and the site of the infusion on the percentage of time spent in the open arms ($F(1,50)=0.035$, $P=0.85$).

In addition, there was no significant difference in general activity levels between the control and midazolam-treated rats as assessed by the total number of arms entered ($F(1,50)=0.253$, $P=0.62$; *Table 3-1*), and the total number of closed arms entered ($F(1,50)=0.355$, $P=0.55$; *Table 3-1*). Finally, there were no significant differences between control and midazolam-treated rats with one or both cannulae located outside of the MPFC on percentage of open arm entries ($t(17)=-0.925$, $P=0.37$) or percentage of open arm time ($t(17)=-0.975$, $P=0.34$).

Finally, to further investigate the possibility of a relationship between the sites of the infusions on the anxiolytic effects observed after midazolam

treatment, the correlation between the depth of the infusion sites and the percentage of open arm entries and time was examined. The overall depth of the infusion sites was calculated by taking the dorsal/ventral position of the bilateral infusion sites from the dorsal surface of the brain. This analysis showed that among rats receiving midazolam infusions, there was no significant correlation between the dorsal/ventral location of the infusions and the percentage of open arm entries ($r=-0.022$, $df=27$, $P>0.05$), and the percentage of time spent in the open arms ($r= 0.154$, $df=27$, $P>0.05$).

3.3. Shock-Probe Burying Test

Fig. 4 depicts the total duration of time spent burying during the shock probe burying test. Rats receiving infusions of midazolam spent significantly less time burying the electrified shock-delivering probe than controls ($F(1,44)=6.826$, $P=0.01$) . There was no significant difference between the scores of rats with dorsal or ventral implants ($F(1,44)=0.007$, $P=0.93$). Also, there was no significant interaction between the drug treatment (vehicle or midazolam) and the site of the infusion (dorsal or ventral) on the total time spent burying the probe ($F(1,44)=0.083$, $P=0.78$).

There was no significant difference between the control and midazolam-treated rats in the number of shocks taken during the 15 minute test ($F(1,44)=0.028$, $P=0.87$; *Table 3-1*), suggesting that working memory was not affected by the drug treatment. In addition, there was also no significant difference in general activity levels between the control and midazolam-treated

rats as assessed by the time spent immobile during testing ($F(1,44)=3.776$, $P=0.06$; *Table 3-1*). The immobility observed primarily consisted of resting and sleep-related behaviour. In addition, there was no significant interaction between the drug treatment (vehicle or midazolam) and the site of the infusion (dorsal or ventral) on immobility ($F(1,44)=2.304$, $P=0.14$; *Table 3-1*). Furthermore, there was no significant difference between control and midazolam-treated rats in their pain sensitivity, as assessed by the shock reactivity rating ($F(1,44)=3.431$, $P=0.07$; *Table 3-1*). In addition, there were no significant differences between the control and midazolam-treated rats with one or both cannula located outside of the MPFC on the time spent burying the shock-probe ($t(16)=-1.349$, $P=0.196$).

Finally, to further investigate the possibility of a relationship between the sites of the infusions on the anxiolytic effects observed after midazolam treatment, the correlation between the depth of the bilateral infusion sites and the time spent burying the shock-probe was calculated. Among rats receiving midazolam infusions, there was no significant correlation between the dorsal/ventral location of infusions and the time spent burying the probe ($r=-0.049$, $df=22$, $P>0.05$).

4. Discussion

The findings of the present study indicate that midazolam infusions into the medial prefrontal cortex (MPFC) attenuate fear-related behaviour as assessed by

the elevated plus-maze and the shock-probe burying tests. In the elevated plus-maze, rats receiving midazolam infusions into the MPFC had a significantly greater percentage of open arm entries, and a greater percentage of time spent in the open arms, than controls receiving infusions of vehicle alone (*Fig. 3-3a, 3-3b*). In the shock-probe burying test, rats receiving midazolam infusions into the MPFC spent significantly less time burying the shock-probe than controls (*Fig. 3-4*). The results from both behavioural tests show that intra-MPFC infusions of midazolam attenuated fear and anxiety. In addition, the results of the present study showed no difference between dorsal and ventral MPFC midazolam infusions in their ability to produce anxiolytic effects in the elevated plus-maze or shock-probe burying tests (*Fig. 3-3a, 3-3b, 3-4*). Therefore, the present findings do not substantiate the notion of a functional difference existing between the dorsal and ventral subregions of the MPFC on fear-related behaviour, at least in regard to the effects of benzodiazepine receptor activation in the dorsal and ventral MPFC. Furthermore, the decrease in fear-related behaviour resulting from midazolam infusions appear to be specific to the MPFC as there was no significant difference between vehicle and midazolam when one or both cannula infusion sites were located outside of the MPFC, on any of the main measures of fear and anxiety in the elevated plus-maze and shock-probe tests (percentage of open arm entries, percentage of open arm time, and total time spent burying the shock-probe).

The finding that intra-MPFC midazolam infusions attenuate fear-related behaviour is strengthened by the lack of any effects of midazolam infusions on a

number of potential confounding variables. Both tests contained measures of general activity and no significant effects due to drug treatment were found in the present study (number of arm entries and time spent immobile; *Table 3-1*). Furthermore, while an anxiolytic profile in the elevated plus-maze involves an increase in open arm activity, anxiolysis in the shock-probe test is represented by a decrease in probe burying. Since the findings of the present study show anxiolytic effects in both the elevated plus-maze and the shock-probe burying tests, the findings cannot be a result of a general increase or decrease in activity levels, or a general inhibition or disinhibition of behavioural responses. In addition, working memory as assessed by the number of shock taken during the shock-probe test was not altered by intra-MPFC infusions of midazolam. Pain sensitivity as assessed by the shock reactivity score recorded in the shock-probe test was also not affected by midazolam infusions. Therefore, the anxiolytic results in the shock-probe burying test did not appear to be the result of decreased pain sensitivity or an impairment in the ability of the rat to form an aversive association between the probe and the electric shock administered. In summary, the results from the behavioural testing indicated that MPFC midazolam infusions produced no significant effects on the potential confounding variables of general activity, memory or pain sensitivity, providing convincing evidence that rats receiving midazolam-treatment show a selective attenuation of fear-related behaviour.

To our knowledge this is first study to examine the effects of intra-MPFC benzodiazepine infusions, or infusions of any $GABA_A$ agonist/antagonist, on fear

and anxiety as assessed by well-validated behavioural assays of anxiety. Previous studies have shown that treating rodents with a variety of benzodiazepines attenuates stress and fear-related increases in dopamine, norepinephrine and Fos-LI [15, 31, 34, 39, 48, 60, 69]. The findings of the present study complement these previous findings by demonstrating that direct activation of the benzodiazepine receptor population of the MPFC specifically decreases fear-related behaviour. These findings suggest that the MPFC may be an important region for mediating the anxiolytic effects of benzodiazepines in rats. In humans, prefrontal cortex benzodiazepine receptors have also been implicated in fear and anxiety. It has been shown through radioligand imaging that benzodiazepine receptor binding is altered in the prefrontal cortex of patients with post-traumatic stress disorder, panic disorder and generalized anxiety disorder [4, 5, 6, 62]. Given the results of the present study and the findings of human research, the MPFC benzodiazepine receptor population may be an important target site for attenuating fear and anxiety-related behaviour associated with anxiety disorders.

The findings of the present study did not reveal any significant difference between dorsal and ventral MPFC midazolam infusions on fear responsivity as assessed by the elevated plus-maze and the shock probe burying test. This result is somewhat surprising considering the functional differences, on fear-related behaviour and cognitive processes, found between the two regions in previous research [37, 66]. In the present study, dorsal infusions were limited to the anterior cingulate and dorsal prelimbic subregions within the MPFC, while

ventral infusions were limited to the infralimbic and ventral prelimbic subregions (*Fig. 3-2*). There was no physical overlap between the areas covered by either infusion site. However, although the dorsal infusion sites were restricted to the dorsal prelimbic and the ventral infusion sites restricted to the ventral prelimbic, both infusion sites did cover the some part of the prelimbic region. Furthermore, it is possible that the midazolam infusions, performed prior to behavioural testing, resulted in different diffusional patterns than infusions of the Chicago blue stain, which were performed prior to histological processing. In addition, since only one dose of midazolam was used, the possibility of the dorsal and ventral MPFC having a differential dose sensitivity to benzodiazepines cannot be ruled out by the present study. Therefore, though the present findings do not corroborate the notion of a functional difference between the dorsal and ventral MPFC, the functional specialization of fear-related processes within the MPFC cannot be ruled out by the current study.

Since midazolam produces neuronal inhibition via the $GABA_A$ receptor complex [13], infusions of midazolam into the MPFC can be viewed as having 'inactivated' the region. Therefore, the results of the present study are consistent with and substantiate the findings from previous research indicating that permanent lesions of the MPFC produce an anxiolytic profile on a variety of behavioural assays of fear and anxiety [19, 33, 54]. The consistency between the findings of present study and MPFC lesion work reinforces the notion that the MPFC is an important structure in the mediation of fear and anxiety.

Intracranial infusions of benzodiazepines have been shown to produce anxiolytic effects when infused directly into a variety of brain regions, such as the hippocampus, amygdala, bed nucleus of the stria terminalis, and septum (for a review see [36]). However, only infusions of benzodiazepines into the septal region have produced a body of results identical to those found in the current study: an increase in open arm activity in the elevated plus-maze, and, a decrease in probe burying in the shock-probe test, with no effect on the number of contact-induced shocks [46, 47]. Studies examining the effects of permanent lesions of the MPFC or septum on fear-related behaviour have also revealed a matching pattern of findings on the elevated plus-maze and the shock-probe burying tests [35, 54, 64]. These parallel findings, in the intracranial infusion experiments and the lesion experiments, suggest that the both the MPFC and the septum may have analogous functions, possibly working in conjunction with respect to the neural mediation of fear and anxiety. Previous research has indicated that the amygdala is necessary for the full expression of fear and stress-induced neurochemical and electrophysiological responses that occur within the MPFC [17, 18, 55], suggesting that the MPFC exerts its effects on fear and anxiety largely through its interactions with the amygdala. However, the evidence described above raises the possibility that the MPFC may also exert some of its effects on fear and anxiety through an interaction with the septum. Future research should examine the possible interaction between the septum and the MPFC with respect to the neural mediation of fear and anxiety.

While the findings of the present study demonstrate that intra-MPFC infusions of a benzodiazepine attenuate fear-related behaviour, further research on GABA and other receptor types in the MPFC is essential. Given the novelty of the findings of the present study, it would be useful to examine the effects of intra-MPFC infusions of a direct $GABA_A$ agonist, or a $GABA_A$ antagonist, on fear-related behaviour. In addition, previous research has suggested that local application of dopamine agonists can alter GABA signaling in the MPFC [52, 68], and intra-MPFC application of GABA agonists/antagonists can alter dopamine levels or release [11, 51]. Given the extensive evidence demonstrating that dopamine levels and turnover increase in response to fearful or stressful stimuli [3, 15, 23, 20, 40, 41, 59, 61], it may be fruitful to examine a possible interaction between the GABA and dopamine receptor populations within the MPFC on fear-related behaviour. Future use of intracranial infusions to directly examine the effects of GABA and monoamine receptor activation/inhibition on fear-related behaviour will likely yield important information on the neurochemical mechanisms underlying the MPFC's role in fear and anxiety.

Figure 3-1. Schematic diagram of coronal rat brain sections illustrating the location of the ventral tip of internal infusion cannulae for all rats included in the present study. Dorsal MPFC infusion sites are marked with an X, while ventral MPFC infusion sites are marked with an O. Cg1= Anterior cingulate area; DP= Dorsal peduncular area; IL= Infralimbic area; PrL= Prelimbic area; M2= Frontal Cortex, Area 2; VO= Ventral orbital area. The atlas plates are adapted from Paxinos and Watson (1986).

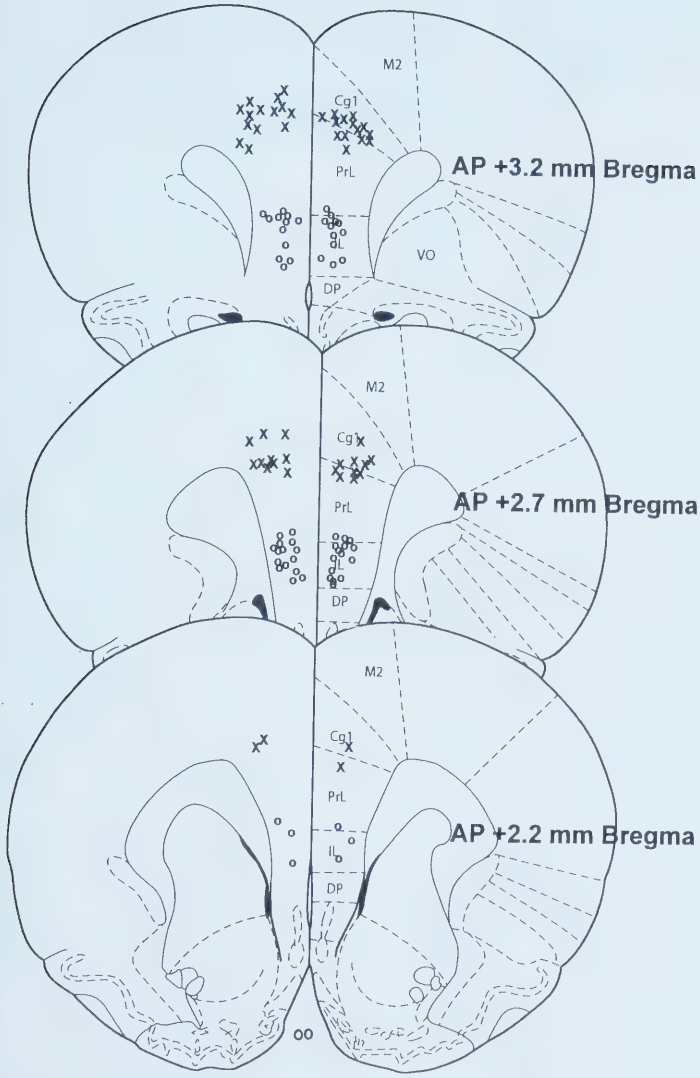

M2
Cg1
PrL
AP +3.2 mm Bregma
IL
VO
DP
M2
Cg1
PrL
AP +2.7 mm Bregma
IL
DP
M2
Cg1
PrL
AP +2.2 mm Bregma
IL
DP

Figure 3-2. Diagram of coronal rat brain sections illustrating the combined area covered by intracranial infusions of Chicago blue stain in 12 rats. Infusions into the dorsal (black) and ventral (grey) MPFC are shown. Cg1= Anterior cingulate area; DP= Dorsal peduncular area; IL= Infralimbic area; PrL= Prelimbic area; M2= Frontal Cortex, Area 2; VO= Ventral orbital area. The atlas plates are adapted from Paxinos and Watson (1986).

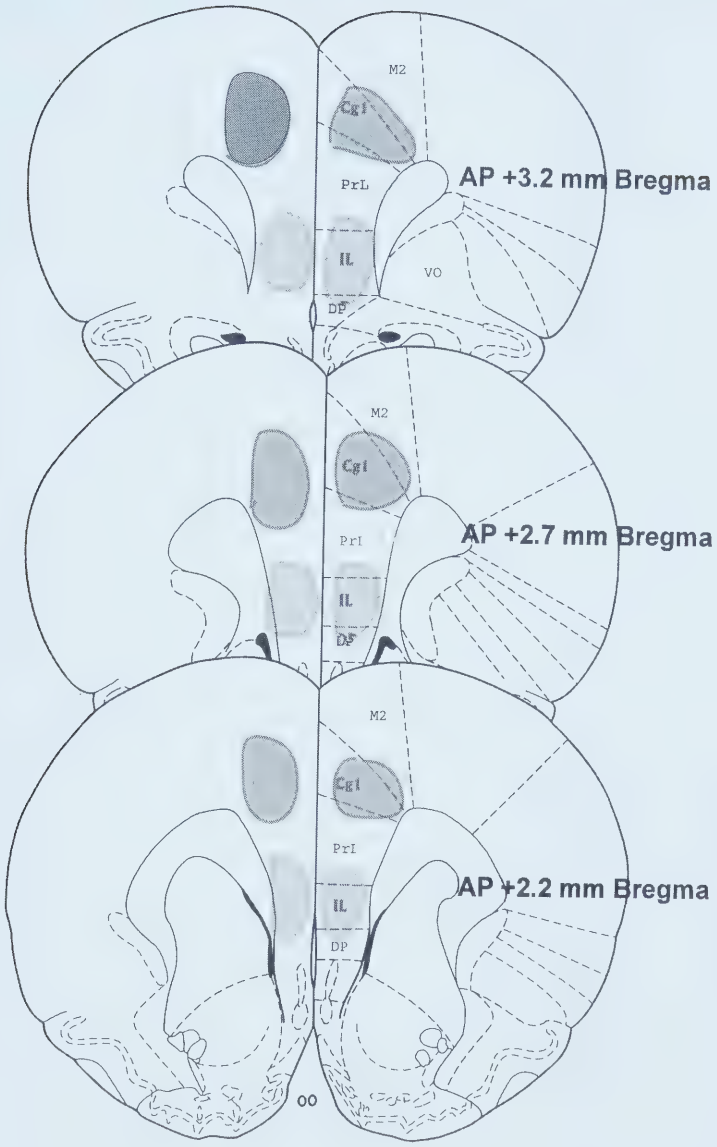
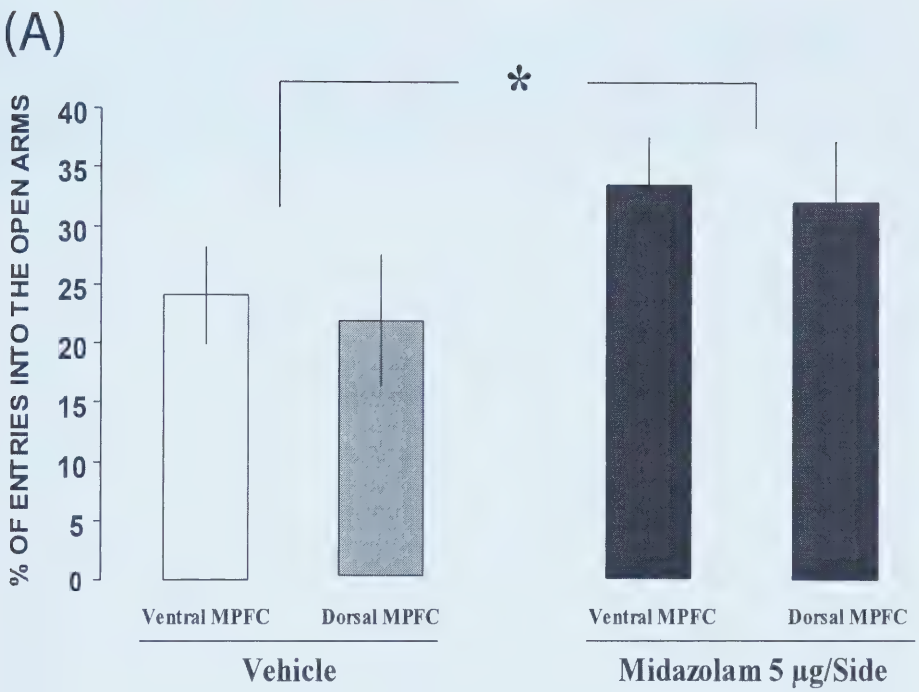

M2
Cg1
PrL
IL
VO
DP
AP +3.2 mm Bregma
M2
Cg1
PrL
IL
DP
AP +2.7 mm Bregma
M2
Cg1
PrL
IL
DP
AP +2.2 mm Bregma

Figure 3-3. (A) Mean (S.E.M.) percent open arm entries in the elevated plus-maze. (B) Mean (S.E.M.) percent open arm time in the elevated plus-maze. The 4 groups depicted in the figure are: dorsal or ventral MPFC infusions of vehicle, and dorsal or ventral MPFC infusions of 5 μg midazolam. * $P < .05$ comparison between the dorsal and ventral MPFC vehicle treated controls versus dorsal and ventral midazolam treated rats.


(A)
% OF ENTRIES INTO THE OPEN ARMS
40
35
30
25
20
15
10
5
0
*
Ventral MPFC
Dorsal MPFC
Vehicle
Ventral MPFC
Dorsal MPFC
Midazolam 5 µg/Side

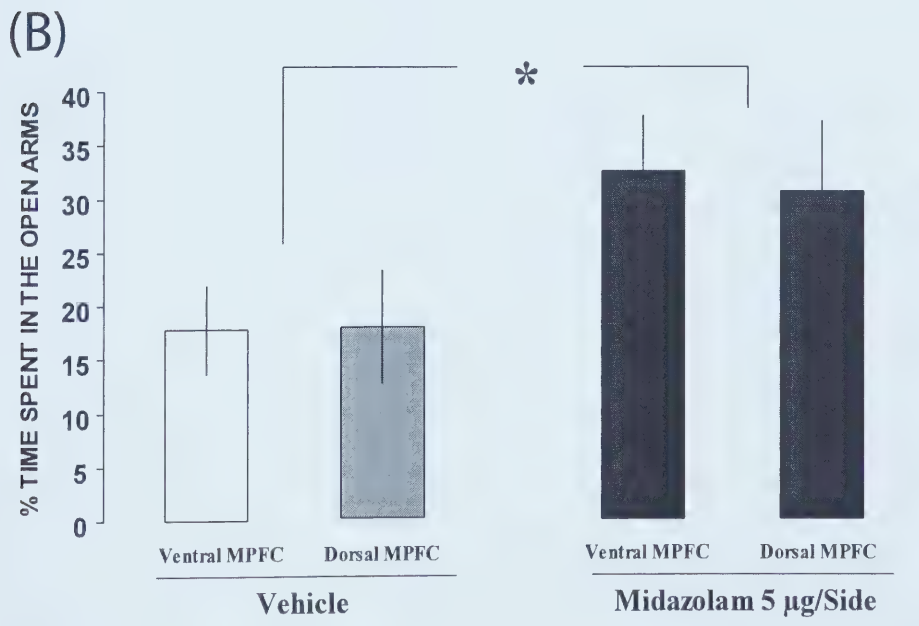

(B)
% TIME SPENT IN THE OPEN ARMS
40
35
30
25
20
15
10
5
0
*
Ventral MPFC
Dorsal MPFC
Vehicle
Ventral MPFC
Dorsal MPFC
Midazolam 5 µg/Side

Figure 3-4. Mean (S.E.M.) total duration of burying during the shock probe test. The 4 groups depicted in the figure are: dorsal or ventral MPFC infusions of vehicle, and dorsal or ventral MPFC infusions of 5 μg midazolam. * $P < .05$ comparison between the dorsal and ventral MPFC vehicle treated controls versus dorsal and ventral midazolam treated rats.

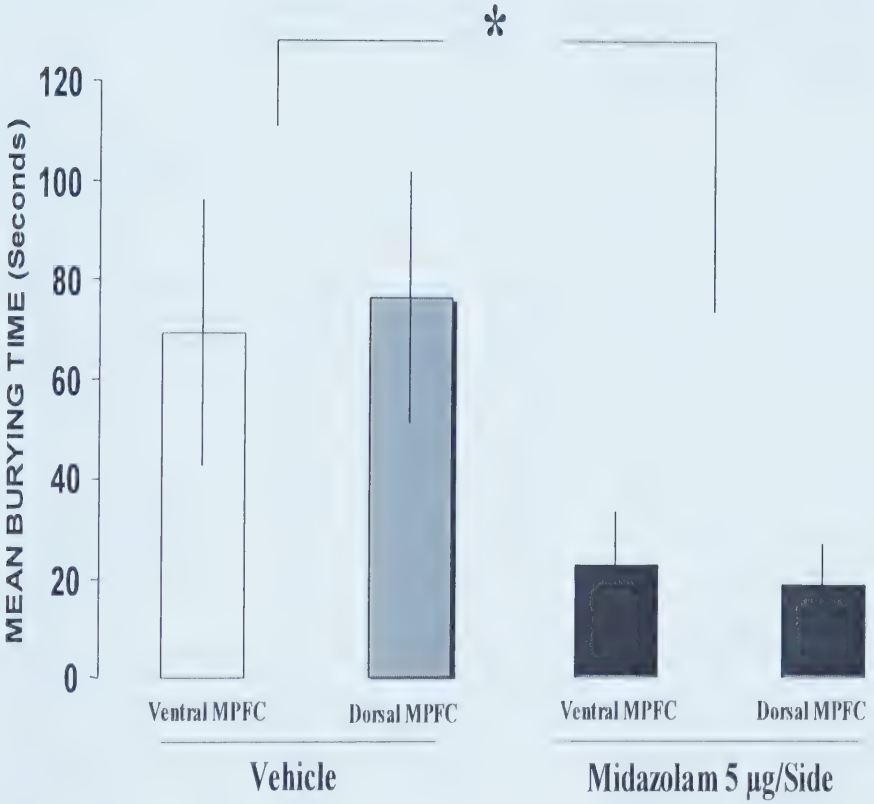

*
MEAN BURYING TIME (Seconds)
120
100
80
60
40
20
0
Ventral MPFC
Dorsal MPFC
Ventral MPFC
Dorsal MPFC
Vehicle
Midazolam 5 µg/Side

Table 3-1. Table entries show the mean (±S.E.M.) number of total arm entries and closed arm entries in the elevated plus-maze. In addition, the table shows the mean (± S.E.M.) number of shocks taken, duration of immobility, and shock reactivity during the shock-probe test.

	Controls (Dorsal)	**Controls (Ventral)**	**Midazolam Treated (Dorsal)**	**Midazolam Treated (Ventral)**
Elevated Plus-Maze				
Number of subjects	11	14	14	15
Number of all arm entries	12.64 ± 1.45	11.21 ± 0.97	12.29 ± 0.92	12.8 ± 1.46
Number of closed arm entries	9.18 ± 1.00	8.64 ± 0.87	8.07 ± 0.67	8.67 ± 1.03
Shock-Probe Burying Test				
Number of subjects	10	14	12	12
Number of shocks taken	3.17 ± 1.00	2.64 ± 0.34	3.08 ± 0.74	2.75 ± 0.41
Mean shock reactivity	2.09 ± 0.10	2.30 ± 0.10	1.91 ± 0.15	2.03 ± 0.12
Duration of immobility	49.00 ± 25.81	4.64 ± 1.58	62.08 ± 34.15	111.17 ± 45.96

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Chapter 4

Inactivation of the Medial Prefrontal Cortex with the $GABA_A$ Receptor Agonist Muscimol Increases Open Arm Activity in the Elevated Plus Maze and Attenuates Shock-Probe Burying in Rats

A version of this chapter has been accepted for publication in *Brain Research.*

1. Introduction

It is well established that $GABA_A$ receptor agonism is an effective means of producing anxiolysis. Previous work has investigated the role of specific brain regions in mediating the anxiolytic effects of $GABA_A$ receptor agonists. Localized microinfusions of benzodiazepines into the amygdala, hippocampus, septum, and ventral tegmental area [5,8,10], and, muscimol infusions (a direct $GABA_A$ receptor agonist) into the amygdala, septum, inferior colliculus, and ventral tegmental area, have been shown to induce anxiolysis [2,5,10,15]. Intracranial infusions of muscimol have also proved useful in determining the effects of general inactivation of a specific brain region [4,22].

Mounting evidence suggests that the medial prefrontal cortex (MPFC) is involved in a number of fear-related processes. For instance, the MPFC has been implicated in stress-induced increases in the release of cortisol, heart rate and blood pressure [1,19]. In addition, exposure to anxiogenic stimuli (e.g., predatory odors) can increase extracellular dopamine levels or turnover in the MPFC [14,18], an effect which is blocked by peripheral administration of benzodiazepines [20]. Furthermore, MPFC lesions are known to affect fear responses in a variety of behavioral tests, including conditioned freezing, shock-probe burying, social interaction, and the elevated plus-maze [6,7,9,16].

Although there is abundant evidence indicating the MPFC is an important structure in the neural mediation of fear-related processes, little research has

directly assessed the role of specific MPFC receptor systems on fear behavior. The prefrontal cortex has a high density of $GABA_A$ receptors, which are composed of apha, beta, and gamma subunits [3,13]. In spite of the ubiquity of $GABA_A$ receptor systems in the cortex, and the known role of $GABA_A$ receptors in fear and anxiety, to date, only one published study has examined the effects of MPFC $GABA_A$ receptor activation on fear behavior. This study showed that intra-MPFC infusions of midazolam, an indirectly acting $GABA_A$ receptor agonist, attenuated the fear-related behavior of rats in the elevated plus-maze and shock-probe burying tests [17]. The purpose of the present study was to corroborate and extend this finding by examining the effects of intra-MPFC infusions of muscimol, a direct $GABA_A$ receptor agonist, on rats' untrained fear reactions in the elevated plus-maze and shock-probe burying tests.

2. Materials and Methods

Subjects were male Sprague-Dawley rats (310-430g; Charles River, St. Constant, Canada), housed individually in polycarbonate cages (47 x 25 x 20.5 cm) and maintained on a 12:12 h light/dark cycle (lights on at 0700 h). Food and water were provided ad libitum. The treatment of all animals conformed to the guidelines of the Canadian Council on Animal Care. The analgesic acetaminophen (120 mg p.o.) was orally administered prior to surgery. An injection of atropine sulfate (0.1mg/0.2ml ip) was administered to reduce

respiratory complications, and the rats were then anaesthetized with pentobarbital (50 mg/kg ip). Subjects were also hydrated with 0.9% saline (3 cc, ip) and treated with penicillin (15,000 IU/0.05 cc im). They were then placed in a stereotaxic apparatus, and an incision was made to expose the skull. Stainless steel 22-gauge guide cannulae (Plastics One, Roanoke, VA) were bilaterally implanted targeting the medial prefrontal cortex (+2.9 mm AP, ±1.9 ML, -1.8 DV; cannulae angled at 15° toward the midline). After surgery rats were given 0.9% saline (3 cc, ip) and placed into a heated environment until recovery from anesthesia.

One week after surgery, intra-MPFC infusions were administered by an infusion pump (Harvard Apparatus 22), at a rate of 0.5 µl/min. Muscimol (Sigma-Aldrich, Oakville, Canada) was dissolved in a solution of phosphate-buffered saline (PBS). Rats were infused with PBS (vehicle) or muscimol (0.175 nmol/0.5 µl or 4 nmol/0.5 µl), through 26 gauge internal infusion cannulae extending 1.2 mm beyond the guide cannulae. While the lower dose of muscimol was selected in order to investigate the specific effects of blockade of $GABA_A$ receptors, the higher dose was primarily selected to approximately equal doses used in GABA-induced neural inactivation studies [4,22]. Infusions were 1 minute in length, with cannulae left in place for an additional minute to allow for diffusion. Behavioral testing commenced 3 minutes after the infusion procedure. Rats were assigned to the same treatment groups for both tests, and were handled by the experimenter for 3 minutes on the 4 days prior to testing.

The elevated plus-maze consisted of two open arms and two enclosed arms (50 x 10 cm), all elevated at a height of 50 cm. The walls of the closed arms were 50 cm in height, and the central square was 10 x 10 cm. Testing was conducted in a quiet and dimly illuminated room (< 15 lux as measured on the surface of the plus-maze), between 1000-1800 h. The variables recorded during the 5 minute test were the number of entries into, and time spent in, the open and closed arms. A rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm. The number of open-arm entries was quantified as a percentage of the total number of entries into all arms (open/total entries x 100). The time spent in the open arms was similarly quantified as a percentage of the total time spent in any arm (open/total time x 100). An increase in the percentage of open-arm entries and open-arm time indicated a decrease in anxiety [11,12]. The number of closed-arm entries served as an index of general activity [11]. One week after plus-maze testing rats were tested in the shock-probe burying paradigm. Testing was conducted between 1000-1800 hr. The apparatus consisted of a Plexiglas chamber (40 x 30 x 40 cm), with woodchip bedding on the floor, and a probe protruding from one end. The probe, 6.5 cm long and 0.5 cm in diameter, was wrapped with two copper wires, which delivered electric current. The current was generated from an AC shocker (H13-15, Colbourn Instruments, Allentown, PA), and the probe was constantly electrified with a 2 mA current. The behavioral test period commenced after the rat received its first, contact-induced shock. During this 15 min test period, the following behavioral measures were recorded: 1) Total time

spent probe-burying; 2) Number of contact-induced shocks from the probe; 3) Amount of time spent immobile (primarily rest or sleep); 4) Reactivity to shock, measured on a 4 point scale for each shock [17]. A mean shock reactivity score was calculated for each rat, and served as a measure of pain sensitivity. The duration of burying was used as the primary measure of fear, with decreases in burying indicating fear-reduction [21].

Following behavioral testing rats were overdosed with chloral hydrate and perfused intracardially with 0.9% (wt/vol) saline, followed by an infusion of 4% (vol/vol) formaldehyde. Subjects' brains were removed and stored in 4% formaldehyde. The brains were cut into 60 μm sections and stained with thionin. The location of the cannulae tips were determined by a "blind" observer. All data were expressed as means and standard errors of the mean (S.E.M.) and analyzed with one-way analysis of variance (ANOVA), followed by least significant difference (LSD) tests of the means of each dose condition (α=0.05).

3. Results

All rats included in present study had infusion sites located in the infralimbic and prelimbic regions (n=54). The behavioural data of rats with either of the bilateral cannulae located outside of the prelimbic and infralimbic regions (n=24) were excluded from the intra-MPFC infusion groups. The

majority of animals with cannulae misplacements had one, or both, cannulae located at a site targeting areas ventral or lateral of the MPFC.

Intra-MPFC infusions of muscimol significantly increased the percentage of open-arm entries ($F(2,50)=3.758$, $P=0.030$; *Fig. 4-1*), and open-arm time ($F(2,50)=5.341$, $P=0.008$; Fig. *4-1*). Comparisons of the different dose conditions revealed that infusions of 0.175 nmol of muscimol significantly increased the percentage of open-arm time when compared to controls, while the 4 nmol dose increased both the percentage of open-arm entries and open-arm time ($P<0.05$). There was an increase in general activity in the muscimol-treated rats as assessed by the number of closed-arm enteries ($F(2,50)=5.750$, $P=0.006$; *Table 4-1*). However, analysis of covariance (ANCOVA) indicated that the number of closed-arm entries did not account for the effects of muscimol on open arm activity (i.e. the open arm activity measures were still significant when the between group variation in general activity was statistically controlled). The ANCOVA showed that the differences between the treatment groups on the percentage of open-arm entries ($F(2,49)=4.517$, $P=0.016$) and the percentage time spent in the open arms ($F(2,49)=3.3920$. $P=0.026$) were significant. In addition, the data from rats with misplaced cannulae revealed no significant differences between muscimol-treated and control animals (percentage of open-arm entries: $F(2,21)=0.444$, $P=0.647$; percentage of open-arm time: $F(2,21)=0.806$, $P=0.460$; Table *4-1*).

Muscimol also decreased the time rats spent burying the probe ($F(2,48)=3.558$, $P=0.036$; Fig. *4-1*). Comparisons of the dose conditions

revealed that infusions of either the 0.175 nmol or 4 nmol dose significantly decreased burying time when compared to control treatment ($P<0.05$). There were no significant differences between the groups on the number of shocks taken ($F(2,48)=1.343$, $P=0.271$; Table *4-1*), time spent immobile ($F(2,48)=0.141$, $P=0.869$; *Table 4-1*), or shock reactivity ($F(2,8)=0.005$, $P=0.995$; Table *4-1*). In addition, analysis of the data from rats with misplaced cannulae revealed no differences between the treatment groups on time spent burying ($F(2,20)=1.161$, $P=0.333$; Table *4-1*).

4. Discussion

The present findings clearly show that bilateral intra-MPFC infusions of muscimol attenuated fear-related responses in the elevated plus-maze and shock-probe burying paradigms. Both doses (0.175 nmol and 4 nmol) significantly increased the percentage of open-arm time, while the higher dose increased the percentage of open-arm entries. In addition, both doses significantly decreased the duration of probe-directed burying in the shock-probe paradigm. These findings indicate that both doses of muscimol are capable of producing anxiolytic effects in two different assays of fear behavior. Furthermore, muscimol had no significant effect on pain sensitivity (i.e. shock reactivity), or passive avoidance of the probe (i.e. number of shocks), indicating that the decrease in burying behavior did not result from analgesia or a failure to associate the electric shock with the probe. While muscimol did increase general

activity in one test (i.e. number of closed-arm entries), analysis of covariance revealed this effect did not account for the muscimol-induced increase in open-arm activity. Furthermore, general activity was not changed in the shock-probe burying test. Finally, there were no significant effects of muscimol treatment on fear-related responses in either test when cannulae did not target the MPFC, suggesting that the anxiolytic effects of the present study were indeed produced by the actions of muscimol on the MPFC.

The present results are consistent with a previous report that intra-MPFC infusions of the indirectly acting $GABA_A$ receptor agonist midazolam attenuates fear-related behaviour in rats [17]. The current study corroborates and extends this finding by demonstrating that the directly acting $GABA_A$ receptor agonist, muscimol, also produces anxiolysis. To further validate these findings it would be constructive to examine the effects of intra-MPFC infusions of $GABA_A$ receptor antagonists, particularly whether a subthreshold dose of a $GABA_A$ receptor antagonist is capable of reversing the anxiolysis produced by stimulating MPFC $GABA_A$ receptors.

The 4 nmol dose of muscimol used in the present experiment is comparable to doses previously used to study the effects of neural inactivation on fear-related behavior [4,22]. Muscimol-induced inactivation of a brain region produces more selective effects than permanent lesions since it likely produces fewer effects on other regions, and is superior to other inactivating agents, such as tetrodotoxin, insofar as it does not inactive of fibers of passage. Given the ubiquity of $GABA_A$ receptors in the rat cortex, the present findings provide

evidence that neural inactivation of the MPFC attenuates fear-related behavior. These findings corroborate previous lesion work which indicates that permanent lesions of the MPFC produce an anxiolytic profile in behavioural assays of fear and anxiety [6,7,16].

The present findings do not appear congruent with the notion that the primary role of the MPFC in fear and anxiety is inhibitory, since inactivation of an inhibitory center would presumably enhance rather than attenuate fear-related behavior. Rather, the results of the present study suggest that the MPFC may contribute to the fundamental neural processing underlying the expression of fear-related behavior.

Figure 4-1. **(A)** Mean (±S.E.M.) percent open arm entries in the elevated plus-maze. **(B)** Mean percent open arm time in the elevated plus-maze. **(C)** Mean duration of shock-probe directed burying during the shock probe test. The 3 treatment groups depicted in the figure are: bilateral intra-MPFC infusions of Vehicle (PBS), Muscimol (0.175 nmol), Muscimol (4 nmol).. * P<0 .05 for comparisons between muscimol treated and control animals.

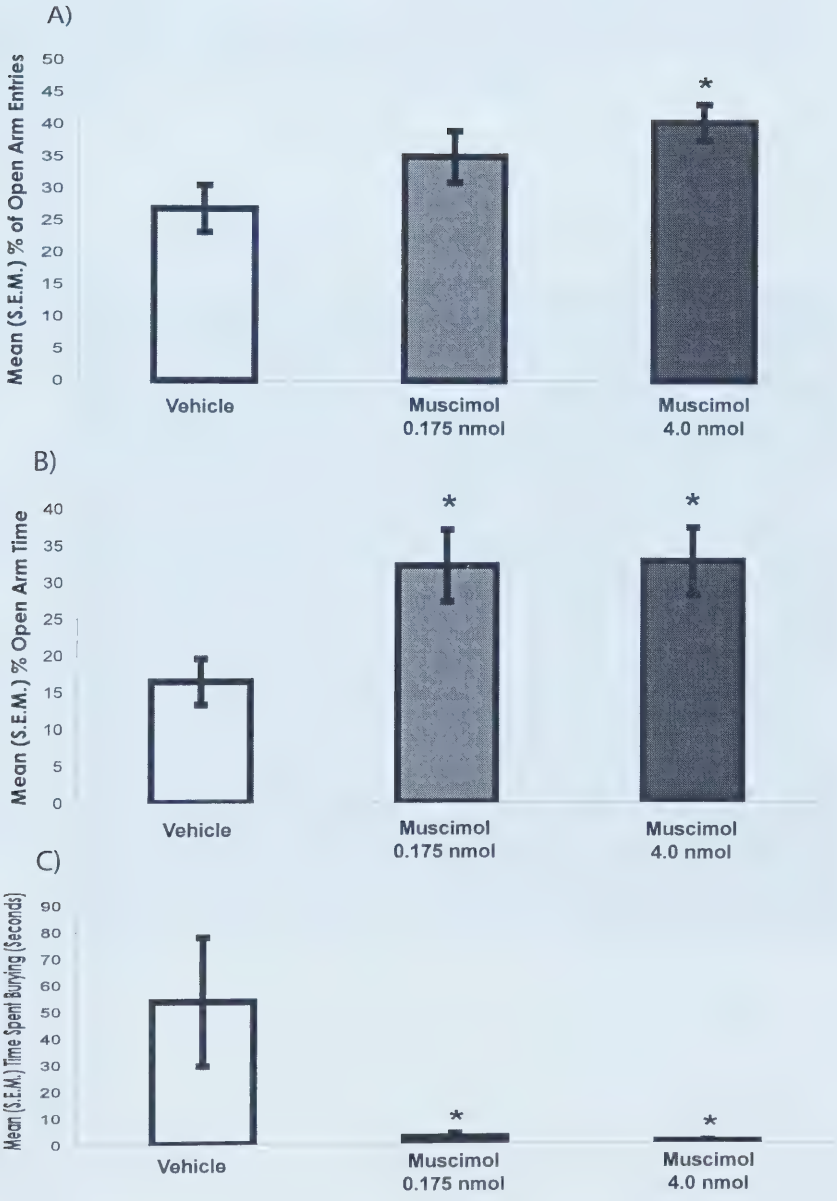
A)
Mean (S.E.M.) % of Open Arm Entries
50
45
40
35
30
25
20
15
10
5
0
*
Vehicle
Muscimol
0.175 nmol
Muscimol
4.0 nmol
B)
Mean (S.E.M.) % Open Arm Time
40
35
30
25
20
15
10
5
0
*
*
Vehicle
Muscimol
0.175 nmol
Muscimol
4.0 nmol
C)
Mean (S.E.M.) Time Spent Burying (Seconds)
90
80
70
60
50
40
30
20
10
0
*
*
Vehicle
Muscimol
0.175 nmol
Muscimol
4.0 nmol

Table 4-1. This table shows the mean (±S.E.M.) values of the following behavioral measures in rats given intra-MPFC infusions: the number of arm entries, number of closed arm entries, number of shocks received, total time spent immobile, and shock reactivity. Also shown are the mean (±S.E.M.) values of the following measures in rats given intracranial infusions located outside of the MPFC: the percentage of open arm entries, percentage of time spent in the open arms, and total time spent burying.

	Elevated Plus-Maze				Shock-Probe Burying			
Treatment Groups	Number of Arm Entries	Number of Closed Arm Entries	*Infusion outside of MPFC:* % Open Arm Entries	*Infusion outside of MPFC:* % Open Arm Time	Number of Shocks Taken	Time Spent Immobile *(Seconds)*	Shock Re-activity	*Infusion outside of MPFC:* Time Spent Burying *(Seconds)*
Vehicle (PBS)	**9.7** (±0.9) ***N= 20***	**6.7** (±0.6) ***N= 20***	**37.4** (±3.6) ***N= 8***	**27.1** (±8.1) ***N= 8***	**1.6** (±0.4) ***N= 20***	**130.7** (±32.0) ***N= 20***	**2.2** (±0.1) ***N= 20***	**26.9** (±11.9) ***N= 7***
Muscimol (0.175 nmol)	**14.9** (±1.0) * ***N= 17***	**9.4** (±0.6) * ***N= 17***	**30.6** (±4.8) ***N= 8***	**17.9** (±4.9) ***N= 8***	**2.7** (±0.4) ***N= 18***	**123.7** (±30.6) ***N= 18***	**2.2** (±0.1) ***N= 18***	**26.0** (±13.2) ***N= 8***
Muscimol (4 nmol)	**15.1** (±1.1) * ***N= 16***	**9.1** (±0.8) * ***N= 16***	**36.5** (±7.4) ***N= 8***	**28.9** (±6.1) ***N= 8***	**3.7** (±0.6) ***N= 13***	**150.4** (±42.4) ***N= 13***	**2.1** (±0.1) ***N= 13***	**6.5** (±6.2) ***N= 8***

5. References

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Chapter 5

Selective Antagonism of Medial Prefrontal Cortex D4 Receptors Decreases Fear-Related Behaviour in Rats

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1. Introduction

A number of studies have shown that the medial prefrontal cortex (MPFC) is involved in fear and stress reactions (e.g. [19,21]). For example, changes in dopamine (DA) activity in the MPFC occur in response to a variety of anxiogenic stimuli. Predatory odours, inescapable footshock, and anxiogenic drugs have all been shown to consistently increase extracellular DA levels or DA turnover in the MPFC [1,7,9], whereas anxiolytic compounds have been shown to attenuate fear and stress-induced increases in MPFC DA activity [22].

Although the responsiveness of mesoprefrontal DA to fear and stress is well established, very little research has directly examined how dopamine receptors in this pathway influence fear-related behaviour. It has been reported that intra-MPFC infusions of DA agonists and antagonists modulate fear-related behaviour in conflict testing, pentylenetetrazol discrimination, and conditioned freezing [3,4,14]. However, non-selective DA ligands were used in these studies, making it difficult to determine which DA receptor sub-types mediated the effects. Furthermore, these behavioral assays of fear and stress depend, to some extent, upon the learning and memory of aversive associations. Since the prefrontal cortex is known to contribute to memory-related processes (for review see [6]), it is difficult in these tests to separate effects on fear from effects on learning and memory. Although it has been reported that intra-MPFC infusions of the selective D2 antagonist sulpiride modulates untrained fear reactions in the

elevated-plus maze [26], to our knowledge no study has examined the effects of selective modulation of other MPFC dopamine receptor subtypes on fear behaviour.

Of particular interest are D4 receptors. D4 receptors have been estimated to be the most common D2-like receptor in the cortex [8], and are known to be densely concentrated in the MPFC. They are localized on GABAergic interneurons as well as the pyramidal cells of rodents and primates [10,28]). In addition, there is evidence that D4 receptors may modulate rodents' untrained fear responses. For example, it has been reported that D4 receptor knockout mice show altered fear reactivity in the elevated plus-maze and the light dark exploration test [5]. These observations suggest that an examination of the role of MPFC D4 receptors in rodents' fear reactions is warranted.

Thus, the present study investigated the effects of intra-MPFC infusions of selective dopamine receptor antagonists (D4, D1 and D2) on rats' fear reactions in the elevated plus-maze and the shock-probe burying tests. Both tests are well-validated models of anxiety, and are effective in detecting the anxiolytic properties of a variety of compounds [23]. The first experiment examined the effects of intra-MPFC infusions of a highly selective D4 antagonist (L-745,870). The second examined the effects of intra-MPFC infusions of a D1 (SCH-23390) or a D2 (remoxipride) receptor antagonist.

2. Materials and Methods

The procedures used in experiment 1 and 2 were identical, with the exception of the different drug treatment groups described in the *intracranial infusion procedure* section.

2.1. Subjects and surgery

The subjects were 172 male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada), weighing 250-430 grams at the time of surgery. Rats were individually housed in polycarbonate cages, maintained on a 12:12 h light/dark cycle (lights on at 0700 h), and provided with food and water ad libitum. The treatment of all animals conformed to the guidelines of the Canadian Council on Animal Care. The subjects were injected with atropine sulfate (0.1mg/0.2ml ip), and were then anaesthetized with sodium pentobarbital (50 mg/kg ip). Subjects were also treated with penicillin (15,000 IU/0.05 cc im) and the analgesic acetaminophen (120 mg po) prior to surgery. The rats were then placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA), and an incision was made to expose the skull. Stainless steel 22-gauge guide cannulae (Plastics One, Roanoke, VA, USA) were bilaterally implanted, targeting the medial prefrontal cortex (+2.9 mm AP, ±1.9 ML, -1.8 DV; all cannulae angled at 15° towards the midline). The cannulae were secured to the skull with four jeweler's screws and cranioplastic cement. Following surgery rats were placed in a heated environment, and were returned to their home cages after waking from anesthesia.

2.2. Intracranial Infusion Procedure

Infusions were administered via an infusion pump (Harvard Apparatus 22, Harvard Apparatus, Holliston, MA, USA), at a rate of 0.5 µl/min. To eliminate the possibility of the drugs interacting with saline or a phosphate buffer, all drugs were dissolved in distilled water. Infusions were made through 26 gauge stainless steel internal cannulae attached by polyethylene tubing to 10 µl Hamilton syringes. The internal cannulae extended 1.2 mm below the guide cannula, and bilateral infusions were conducted simultaneously. The cannulae were left in place one minute after the end of the infusions to allow for diffusion. In experiment 1 rats were bilaterally infused with one of the following four solutions: vehicle, or L-745,870 trihydrochloride (0.2, 1, or 10 nmol; Tocris Cookson). L-745,870 is a highly selective D4 antagonist (> 2000-fold affinity over other DA receptors; [2]). In experiment 2 rats were bilaterally infused with one of the following six solutions: vehicle (distilled water), R(+)-SCH-23390 (0.2, or 1 nmol; Sigma, St. Louis, MO, USA), or remoxipride hydrochloride (0.2, 1, or 10 nmol; Tocris Cookson, Ellisville, MO, USA). SCH-23390 is a highly selective D1 antagonist (> 3000-fold affinity over D2-like receptors), while remoxipride is selective for D2 receptors (>9-fold affinity over D4 receptors; [11,18]). The doses of the dopamine antagonists used in the present study were chosen based on the range of effective intra-MPFC doses of dopamine antagonists used in previous research (e.g. [15,26]). A 10 nmol dose of SCH-23390 was not used because it exceeded the maximum solubility of the drug when dissolved in 0.5 µl of water.

2.3. Elevated Plus-Maze Test

Plus-maze testing commenced at a minimum of 7 days following surgery. Rats were handled for 3 minutes on each of the 4 consecutive days prior to testing. Testing began 3 minutes after the end of the infusion procedure. The maze was a wooden plus-shaped apparatus, and elevated at a height of 50 cm. It consisted of two 50 x 10 cm open arms, and two 50 x 10 x 50 cm enclosed arms, all arms having an open roof. Testing was conducted in a quiet and dimly illuminated (< 15 lux) room. All testing occurred between 0900-1800 h, and behaviour was recorded for 5 minutes. The maze was cleaned with distilled water after each rat was tested. Four variables were measured during each 5 minute test: 1) Time spent in the open arms. 2) Time spent in the closed arms. 3) Number of entries into the open arms. 4) Number of entries into the closed arms. A rat was considered to have entered or spent time in an arm only if all four paws were in the arm. A percentage was calculated from the time spent in the open arms, open arm time/total arm time x 100. A similar percentage was calculated from the number of open arm entries, open arm entries/total arm entries x 100. A higher percentage of open arm time or open-arm entries were taken as measures of anxiolysis [12]. In addition, the total number of closed arm entries was used as an index of general activity [12,16].

2.4. Shock-Probe Burying Test

One week after plus-maze testing, rats were tested in the shock-probe apparatus. Because previous work has shown no evidence of drug carry-over effects after a one week period [24], rats were placed in the same drug treatment groups for both behavioral tests. The shock probe apparatus consisted of a 40 x 30 x 40 cm Plexiglas chamber evenly covered with woodchip bedding. An electrified probe protruded from one side of the chamber, 2 cm above the bedding. The probe (6.5 cm length, 0.5 cm diameter) was wrapped with copper wire though which an electric current was administered. The current was generated by an AC shocker (H13-15, Colbourn Instruments, Allentown, PA, USA). All testing occurred between 0900-1800 h. Testing commenced with the rat being placed in the apparatus facing away from the shock–probe. Rats contacting the probe received a 2 mA shock. A 15 minute observation period began once the first shock was delivered and the probe remained electrified throughout the test. The following four measures were recorded: 1) Total amount of time spent spraying bedding material towards the shock probe with the forepaws (i.e. burying behavior). 2) Number of shocks received due to contact with the probe, taken as a measure of aversive learning. 3) Amount of time spent immobile (e.g. rest), taken as a measure of general activity. 4) Reactivity to shock, in which each shock taken during testing was measured on a 4 point scale (1= head or paw flinch, 2= whole body flinch, 3= whole body flinch followed by immediate ambulation away from probe, 4= whole body flinch followed by rapid ambulation away from probe) [13]. A mean shock reactivity score was calculated for each rat and was used to assess pain

sensitivity. The total duration of burying was used as the primary measure of fear behavior, with decreases in the duration of burying indicating a reduction in fear [25].

2.5. Histology

Upon completion of testing, rats were overdosed with chloral hydrate and perfused intracardially with 0.9% (wt/vol) saline followed by an infusion of 4% (vol/vol) formaldehyde. Subjects' brains were then removed and placed in a postfix solution of 4% formaldehyde for at least 48 hours. The brains were cut into 60 μm sections, and every second section was mounted onto glass slides and stained with thionin.

2.6. Statistical Analyses

The results from the elevated plus-maze test were expressed as means and standard errors of the mean (S.E.M.). Behavioural measures from the plus-maze were analyzed with one-way analysis of variance (ANOVA; α=0.05). Post hoc LSD tests were used to analyze the effects of drug treatment compared to the control condition (α=0.05). Because the burying data were not normally distributed, they were analyzed using non-parametric tests (Kruskal-Wallis and Mann-Whitney U-tests; α=0.05), and expressed as medians with interquartile ranges (IQ). Eight animals could not be tested in the burying apparatus because the cannulae implants had become unstable during the period between the two behavioral tests.

3. Results

3.1. Histology

Rats included in the analysis had infusion sites located in the prelimbic and infralimbic subregions of the MPFC. Thirty-six rats had cannulae located outside of the prelimbic and infralimbic regions, and were therefore excluded from the intra-MPFC infusion groups. However, the data from these excluded rats were analyzed separately to determine the site specificity of any treatment effects. Six rats showed substantial amounts of necrosis, and were therefore excluded from all behavioural analysis. Overall, there was no noticeable difference between the location of cannulae in experiment 1 and 2.

3.2. Elevated Plus-Maze Test

The results of Experiment 1 showed that intra-MPFC infusions of L-745,870 had a significant effect on the percentage of open-arm entries *($F(3,49)=3.37$, $P=0.026$; Figure 5-1; Table 5-1*), and the percentage of open-arm time *($F(3,49)=3.20$, $P=0.031$; Figure 5-1; Table 5-1).* Post hoc analyses revealed that the open-arm exploration of animals receiving the 1 nmol dose of L-745,870 was significantly higher than that of controls *($P<0.05$),* suggesting that the 1 nmol dose produced anxiolysis in the elevated plus-maze. There was no significant difference in general activity between the groups as assessed by

the number of closed arms entered *(F(3,49)=0.74, P=0.53; Table 5-1).* In addition, analysis of the data from rats with misplaced cannulae revealed no significant differences between rats given the vehicle or rats treated with L-745,870 (percentage of open-arm entries: *F(3,15)=0.66, P=0.59;* percentage of open-arm time: *F(3,15)=1.21, P=0.34).*

The results from Experiment 2 revealed no differences between the control, remoxipride, or SCH-23390 treatment groups on any measure in the elevated plus-maze. There were no significant differences between the groups on the percentage of open-arm entries *(F(5,71)=0.69, P=0.63; Table 5-1),* the percentage of open- arm time *(F(5,71)=0.68, P=0.64; Table 5-1)* or the number of closed-arm entries *(F(5,71)=0.56, P=0.73; Table 5-1).*

3.3. Shock-Probe Burying Test

The results of Experiment 1 showed that intra-MPFC infusions of L-745,870 had a significant effect on the median time the rats spent burying the probe *(U(3,46)=8.15, P=0.043; Figure 5-1; Table 5-1).* Individual Mann-Whitney U-tests revealed that animals receiving either the 0.2 nmol or the 1 nmol dose of L-745,870 buried the probe significantly less than vehicled-treated controls *(P<0.05),* suggesting that both doses of L-745,870 produced anxiolysis in the shock-probe test. There were no significant differences between the groups on the number of shocks taken *(U(3,46)=1.11, P=0.78; Table 5-1),* time spent immobile *(U(3,46)=1.97, P=0.58; Table 5-1),* or shock reactivity *(U(3,46)=1.61, P=0.66; Table 5-1).* In addition, analysis of the data from rats

with misplaced cannulae revealed no overall difference between rats given vehicle or drug on time spent burying the probe *(U(3,14)=0.23), P=0.97).*

The results from Experiment 2 revealed no differences between the control, remoxipride, or SCH-23390 treatment groups on any measure in the shock-probe test. There were no significant differences between the groups on the median time spent burying the shock-probe *(U(5,66)=3.34, P=0.65; Table 5-1),* the number of shocks received *(U(5,66)=1.24, P=0.94; Table 5-1),* time spent immobile *(U(5,66)=5.05, P=0.41; Table 5-1),* or shock reactivity *(U(5,66)=8.47, P=0.13; Table 5-1).*

4. Discussion

The present findings clearly indicate that intra-MPFC infusions of specific doses of the D4 antagonist L-745,870 produce a behavioural profile indicative of anxiolysis. Bilateral infusions of a 1 nmol dose of L-745,870 increased the percentage of open-arm entries and open-arm time in the plus-maze, and the 0.2 and 1 nmol doses decreased the duration of shock- probe induced burying. To our knowledge, this is the first study to demonstrate that intra-MPFC infusion of a selective D4 antagonist is capable of attenuating fear-related behaviour. This finding is reinforced by the absence of any effects on a number of potentially confounding variables. Both tests included measures of general activity (i.e., number of closed-arm entries and immobility), and no

effects due to drug treatment were found. Pain sensitivity, as assessed by shock reactivity, was also not affected by L-745,870. There were no significant differences between the groups in their ability to avoid the shock-probe during their initial test (see number of shocks, Table 1), or during a retention test conducted 9 days later (data not shown). These findings suggest that memory processes related to shock-probe avoidance were not affected by the drug treatment. In summary, L-745,870 produced no observable effects on general activity, memory, or pain sensitivity, indicating the antagonist selectively attenuated fear-related behavior. Furthermore, the present results suggest that the anxiolytic effects of L-745,870 may be restricted to the MPFC, since rats given infusions of L-745,870 outside of this region (i.e. misplaced cannulae) did not show any decrease in fear-related responses.

Previous work has shown that 'knockout' mice lacking D4 receptors display an increase in unconditioned fear responses [5], while the present study has shown that D4 receptor antagonism attenuates fear-related behaviour. This apparent discrepancy could be a result of procedural differences. Removal of genes necessary to produce D4 receptor deficient mice is likely to have a variety of effects unrelated to simple attenuation of D4 receptor activity. Furthermore, while the knockout study targeted all D4 receptors, in the present study *MPFC* D4 receptors were selectively blocked, suggesting anxiolyis may result from the specific inhibition of *MPFC* D4 receptors. Nonetheless, the findings of the studies are complementary with regard to demonstrating the importance of D4 receptors in the neural mediation of fear behaviour.

It is curious that the highest dose of L-745,870 used in the present study was ineffective, whereas the lower doses were anxiolytic. Although non-linear dose-effect functions are often related to the activity of autoreceptors, these receptors appear to have minimal influence over the regulation of prefrontal dopamine activity [17]. Another possibility is that the highest dose of L-745,870 influenced non-D4 receptors, which counteracted the anxiolytic effects induced by D4 antagonism. Given that higher doses increase the possibility of non-selective effects, the lack of a treatment effect at the highest dose actually strengthens the case that *selective* antagonism of D4 receptors decreases fear behaviour.

The present results failed to demonstrate any effect of intra-MPFC infusions of a D1 (SCH-23390) or D2 (remoxipride) antagonist. The latter finding is somewhat unexpected given a previous report that sulpiride, a D2 antagonist, increased the open-arm activity of CD-1 mice in the elevated plus-maze [26]. This discrepancy may be related to species differences, or to differences in the efficacy of these D2 antagonists [11]. It is also possible that remoxipride induced anxiolysis may not have been detected in the shock probe test because of the relatively low levels of probe burying behaviour observed in control animals. However, remoxipride treated rats showed no trend towards increased open arm activity, making undetected anxiolysis less likely. In any case, the findings of the present study are unambiguous in demonstrating the anxiolytic effects of selective antagonism of MPFC D4 receptors.

Intriguingly, MPFC D4 receptors are also known to indirectly modulate GABAergic signaling in prefrontal cortex pyramidal neurons [27]. Benzodiazepines such as midazolam are indirect $GABA_A$ agonists and are known to inhibit MPFC DA responsivity to stressors [22], and to attenuate fear-related behaviour when infused into the MPFC [20]. In combination, these findings raise the possibility that MPFC D4 receptors influence fear behaviour through the modulation of MPFC GABAergic activity. Further research examining the potential interaction between MPFC D4 and $GABA_A$ receptors on fear-related behaviour may therefore prove to be fruitful.

Figure 5-1. (A) Mean (±S.E.M.) percent open arm entries in the elevated plus-maze. (B) Mean percent open arm time in the elevated plus-maze. (C) Median (I.Q. range) duration of shock-probe directed burying during the shock probe test

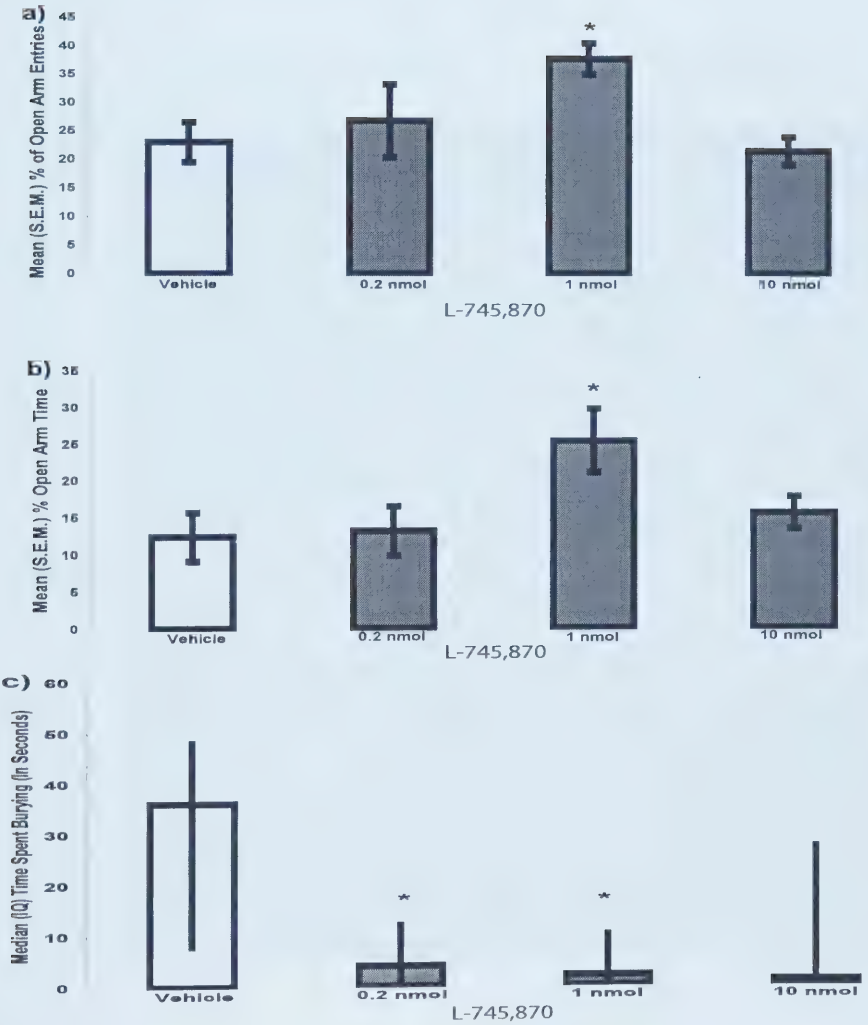
a)
Mean (S.E.M.) % of Open Arm Entries
45
40
35
30
25
20
15
10
5
0
*
Vehicle
0.2 nmol
1 nmol
10 nmol
L-745,870
b)
Mean (S.E.M.) % Open Arm Time
35
30
25
20
15
10
5
0
*
Vehicle
0.2 nmol
1 nmol
10 nmol
L-745,870
c)
Median (IQ) Time Spent Burying (In Seconds)
60
50
40
30
20
10
0
*
*
Vehicle
0.2 nmol
1 nmol
10 nmol
L-745,870

Table 5-1. All behavioural measures from the elevated plus-maze (dark gray), displays the mean (S.E.M) value of each group. All measures from the shock-probe burying test (light gray) display the median (I.Q. range) value. ** Represents a significant difference between treatment and vehicle group ($P < 0.05$).

	Elevated Plus-Maze			Shock-Probe Burying			
Experiment/ Groups	% Open Arm Entries (Mean)	% Open Arm Time (Mean)	Number of Closed Arm Entries (Mean)	Time Spent Burying (Median, Seconds)	Number of Shocks Taken (Median)	Time Spent Immobile (Median, Seconds)	Shock Reactivity (Median)
L-745,870							
Vehicle	**22.9** (3.5) *N= 14*	**12.4** (3.3)	**8.2** (1.0)	**36.0** (40.0) *N= 13*	**2.0** (2.0)	**26.0** (42.0)	**2.0** (0.3)
L-745,870 (0.2 nmol)	**26.8** (6.4) *N= 13*	**13.1** (3.3)	**7.6** (1.2)	**4.0** (12.0) ** *N= 13*	**2.0** (1.0)	**37.0** (65.0)	**2.0** (0.3)
L-745,870 (1 nmol)	**40.0** (2.7) ** *N= 14*	**25.5** (4.3) **	**7.0** (0.7)	**2.0** (10.0) ** *N= 14*	**2.0** (0.8)	**16.5** (117.0)	**2.0** (0)
L-745,870 (10 nmol)	21.6 (2.4) *N= 12*	**15.6** (2.2)	**8.8** (0.5)	**1.0** (27.3) *N= 10*	**2.0** (2.0)	**9.5** (13.0)	**2.0** (0.6)
SCH-23390/ Remoxipride							
Vehicle	**41.1** (4.4) *N= 13*	**30.8** (4.4)	**6.1** (0.9)	**3.0** (11.0) *N= 13*	**3.0** (2.0)	**51.0** (120.0)	**2.0** (0.9)
SCH-23390 (0.2 nmol)	**37.1** (2.2) *N= 13*	**23.8** (4.5)	**6.2** (0.7)	**6.0** (13.0) *N= 13*	**3.0** (2.0)	**35.0** (64.0)	**2.0** (0.3)
SCH-23390 (1 nmol)	**37.1** (6.5) *N= 14*	**26.6** (8.1)	**5.5** (0.9)	**3.0** (42.0) *N= 12*	**3.0** (1.3)	**170.0** (190.8)	**1.7** (0.5)
Remoxipride (0.2 nmol)	**32.1** (5.1) *N= 14*	**21.2** (4.6)	**6.5** (0.8)	**1.0** (27.8) *N= 10*	**2.0** (2.5)	**28.5** (25.5)	**2.0** (0.1)
Remoxipride (1 nmol)	**30.6** (5.0) N= 14	**24.5** (4.9)	**7.0** (0.6)	**16.5** (57.3) *N= 14*	**2.0** (1.0)	**26.0** (75.5)	**2.0** (0.5)
Remoxipride (10 nmol)	**31.3** (6.2) *N= 10*	**31.3** (6.2)	**7.2** (0.8)	**1.5** (12.3) *N= 10*	**3.0** (1.8)	**48.0** (153)	**2.0** (0.2)

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Chapter 6

Effects of Medial Prefrontal Microinfusions of the Dopamine Agonist Apomorphine on Fear-Related Behaviour and General Activity

1. Introduction

Several different lines of convergent research have provided compelling evidence that the prefrontal cortex is an important structure in the neural architecture mediating emotional processes. Fear, anxiety, and stress are among the types of affective experience that have been shown to have robust links to neural activity in the prefrontal cortex. For instance, several imaging studies have revealed atypical patterns of blood flow, associated with neural activity, in the prefrontal cortices of subjects with generalized anxiety disorder, obsessive compulsive disorder, post-traumatic stress disorder, social phobia, panic disorder, high trait anxiety, and depression [5,23,33,40,48,73,77]. In addition, 'normal' subjects exposed to a variety of anxiety-provoking stimuli exhibit prefrontal blood flow and neurochemical changes in a variety of prefrontal cortical regions [27,38,39,68]. Animal studies have also provided a large corpus of research findings suggesting the prefrontal cortex, particularly the medial prefrontal (MPFC) region, is important for the neural mediation of fear, anxiety, and stress. For example, MPFC lesions have been shown to disrupt normal fear-related responses in a diverse array of behavioural assays. MPFC lesions have been reported to produce anxiolytic-like effects in the elevated plus-maze, social interaction, shock-probe burying, open field, and food neophobia tests [8,26,35,36,67,70]. In addition, Fos expression increases in the MPFC of rodents exposed to anxiety-provoking stimuli [41,43], and MPFC activity has

also been strongly associated with extinction of fear conditioning [42,60,61]. Furthermore, the MPFC is also known to modulate anxiety-related physiological responses such as HPA axis activation, and, increased heart rate and blood pressure [13,57,69].

The activation of mesocortical dopamine (DA) by stressors is another empirical phenomenon which has implicated the prefrontal cortex in fear and anxiety. The prefrontal cortex (as well as the nucleus accumbens, amygdala, and other limbic sites) receives extensive dopaminergic projections from the ventral tegmental area (VTA), and the prefrontal cortex also projects back to DA neurons in the VTA [11,20]. A considerable number of research findings have revealed that the VTA DA neurons innervating the prefrontal cortex, in particular the MPFC, are preferentially active when rodents have been exposed to a wide range of fear, anxiety or stress-provoking stimuli. For example, it has been reported that inescapable electric foot-shock, tail pinch, food deprivation, restraint stress, a novel environment, and moderate aversively conditioned stimuli increase extracellular DA levels or DA turnover in the MPFC of rodents, with smaller or no significant changes in other regions receiving DA innervation such as the nucleus accumbens, striatum and even the amygdala [9,10,12,30,32,74,75]. Several studies have also demonstrated MPFC DA activation to different anxiety-provoking stimuli such as predatory odours, social defeat stress, and systemic administration of anxiogenic drugs [4,44,71,76]. Furthermore, it has been shown that the fear and anxiety-induced increase in

MPFC DA activity in rodents can be blocked by acute or chronic treatment with anxiolytic compounds [14-16,31,72].

Despite the substantial body of empirical evidence indicating that the prefrontal cortex, and in particular mesocortical DA, is involved in fear and anxiety, relatively few studies have directly examined the role of prefrontal DA on fear and anxiety-related behaviour. Moreover, the small number of published studies that have investigated this matter provide a somewhat incongruous pattern of findings. Espejo and colleagues have reported that permanent MPFC DA depletion produced by 6-hydroxydopamine, generated an anxiogenic behavioural profile in the elevated plus-maze and light-dark tests [21,22]. However, in another study Broersen and colleagues investigated anxiety-related responses in a modified Geller type conflict procedure, reporting that intra-MPFC infusions of the DA agonist apomorphine attenuated conflict responding, while the DA receptor antagonist *cis*-flupenthixol increased conflict responding, suggesting an anxiogenic profile produced by DA receptor stimulation and an anxiolytic profile produced by DA receptor blockade [7]. Intra-MPFC infusions of these two DA drugs produced a similar anxiolytic and anxiogenic profile during pentylenetetrazol (PTZ) discrimination testing, but no effects for the DA drugs were found for chlordiazepoxide discrimination [6]. Other findings have also suggested that blockade of specific DA receptor types within the MPFC induces anxiolysis in rodents; intra-MPFC infusions of the D2 antagonist sulpiride attenuates fear-related behaviour in the elevated plus-maze (EPM) [83], and the D4 antagonist L-745,870 produces an anxiolytic profile in the EPM and

the shock-probe burying test [66]. On the other hand, Wall and colleagues have also reported intra-MPFC infusions of the D2 agonist quinpirole fails to produce a clear anxiogenic-like effect [83], while the D1 agonist SKF-81297 actually produces an *anxiolytic* profile in the EPM [82]. Finally, intra-MPFC infusions of the DA agonist D-amphetamine, and the DA antagonist *cis*-flupenthixol, were *both* shown to attenuate expression, but not acquisition, of conditioned freezing, suggesting both drugs are capable of producing anxiolysis [53].

Overall, while the findings from these behavioural studies clearly indicate that MPFC DA significantly influences fear and anxiety-related behaviour, the specific effects of MPFC DA receptor activation appear to yield somewhat contradictory findings [6,7,82,83]. In order to further investigate the effects of MPFC DA activation on fear/anxiety behaviour, the present study examined the effects of intra-MPFC infusions of the dopamine agonist apomorphine on behavior in the elevated plus-maze and the shock-probe burying tests. Both tests are well-validated models of fear and anxiety that are known to be effective in detecting the anxiolytic properties of a variety of compounds [78].

2. Materials and Methods

2.1. Subjects

The subjects were 68 male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada), weighing 300-450 grams at the time of surgery. Rats were individually housed in polycarbonate cages (47 x 25 x 20.5 cm) for the

duration of the experiment. They were maintained on a 12:12 h light/dark cycle, with lights on at 0700 h, and were provided with food and water ad libitum. The treatment of all animals conformed to the guidelines of the Canadian Council on Animal Care.

2.2. Surgery

The subjects were injected with atropine sulfate (0.1mg/0.2ml ip), and were then anaesthetized with sodium pentobarbital (50 mg/kg ip). Subjects were also treated with penicillin (15,000 IU/0.05 cc im) and the analgesic acetaminophen (120 mg po) prior to surgery. The rats were then placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA), and an incision was made to expose the skull. Stainless steel 22-gauge guide cannulae (Plastics One, Roanoke, VA, USA) were bilaterally implanted, targeting the medial prefrontal cortex (+2.9 mm AP, ±1.9 ML, -1.8 DV; all cannulae angled at 15° towards the midline). The cannulae were secured to the skull with four jeweler's screws and cranioplastic cement. Following surgery rats were placed in a heated recovery cage, and were returned to their home cages after waking from anesthesia.

2.3. Intracranial Infusion Procedure

Infusions were administered via an infusion pump (Harvard Apparatus 22, Harvard Apparatus, Holliston, MA, USA), at a rate of 0.5 µl/min. Since apomorphine precipitated out of solution in phosphate-buffered saline, all

apomorphine doses were dissolved in distilled water. All solutions had an approximately equivalent pH value since the pH of the vehicle, and the solutions containing the two lower doses of apomorphine, were adjusted to equal the pH of the solution with the highest dose of apomorphine. Infusions were made through 26 gauge stainless steel internal cannulae attached by polyethylene tubing to 10 µl Hamilton syringes. The internal cannulae extended 1.2 mm below the guide cannula, and bilateral infusions were conducted simultaneously. The cannulae were left in place one minute after the end of the infusions to allow for diffusion of the solution. The animals were given bilateral infusions of one of the following four solutions: vehicle (distilled water), apomorphine (1 µg/0.5 µl, each hemisphere), apomorphine (3 µg/0.5 µl, each hemisphere), apomorphine (6 µg/0.5 µl, each hemisphere). The doses of the apomorphine used in the present study were selected based on previous research indicating effective intra-MPFC doses of apomorphine in behavioural assays of anxiety [6,7,53].

2.4. Behavioural Testing

All behavioural testing was recorded on videotape. Behavioural testing occurred at least 7 days following surgery. All rats were handled for 3 minutes, and the cannulae tracts were checked for blockage, on the 4 consecutive days prior to the start of behavioural testing. On testing days, rats were moved from their vivarium to a holding room, separate from the behavioural testing rooms, and were allowed to habituate to the holding room for at least 30 minutes prior to testing. Testing commenced 3 minutes after the end of the intracranial infusion

procedure. Rats were placed in the same treatment groups for both behavioural tests.

2.4.1. Elevated Plus-Maze Test

The maze was a plus-shaped apparatus, constructed from wood, and was elevated to a height of 50 cm. It consisted of two 50 x 10 cm open arms, and two 50 x 10 x 50 cm enclosed arms, all arms having an open roof. Testing was conducted in a quiet room that was dimly illuminated (< 15 lux as measured on the surface of the plus-maze). All testing occurred between 1000-1800 h and behaviour was recorded for 5 minutes. The maze was cleaned with distilled water after each rat was tested. Four variables were measured during each 5 minute test: 1) Time spent in the open arms. 2) Time spent in the closed arms. 3) Number of entries into the open arms. 4) Number of entries into the closed arms. A rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm. The time spent in the open arms was quantified as a percentage of total arm activity, i.e. the amount of time that the rat spent in the open arms relative to the total amount of time spent in all of the arms (open/total time x 100; [50,51]). The number of open arm entries was similarly quantified as a percentage of the number of total arm entries (open/total entries x 100; [50,51]). A higher percentage of open arm time or open-arm entries were taken as measures of anxiolysis [50,51]. In addition, the total number of closed arm entries, and, the total number of all arm entries, were recorded to assess the effects of intra-MPFC apomorphine on general activity [50,59].

2.4.2. Shock-Probe Burying Test

One week after elevated plus-maze testing, rats were tested in the shock-probe burying paradigm. The shock-probe apparatus consisted of a 40 X 30 X 40 cm Plexiglas chamber, evenly covered with woodchip bedding material, with an electrified probe protruding from one side of the chamber 2 cm above the bedding. The electrified probe, or 'shock-probe', was 6.5 cm long and 0.5 cm in diameter, and was helically wrapped with two copper wires though which an electric current was administered. The electrical current was generated from an AC shocker (H13-15 precision regulated shocker, Colbourn Instruments, Allentown, PA, USA). Rats were habituated individually in an identical Plexiglas chamber without the shock-probe for 45-90 minutes daily on the 5 consecutive days prior to shock-probe testing. All testing occurred between 1000-1800 h. At the start of shock-probe testing, rats were individually placed in a corner of the testing chamber facing away from the shock–probe. The probe was electrified and rats coming into contact with the probe received a 2 mA shock. The 15 minute observation period began once the first shock was administered and the probe remained electrified throughout the entire testing period. The following four behavioural measures were recorded: 1) Total amount of time spent moving bedding material towards and on top of the shock probe with rapid alternating movement of the forepaws (i.e. burying behaviour), taken as the primary measure of fear-related behaviour; 2) Number of shocks taken due to contact with the probe, used as a measure of aversive

learning/memory; 3) Amount of time spent immobile (primarily rest and sleep), taken as a measure of general activity; 4) Reactivity to shock, which was measured on a 4 point scale (1= flinch involving head or paw, 2= whole body flinch with or without slow ambulation away from probe, 3= whole body flinch or jumping followed by immediate ambulation away from probe, 4= whole body flinch or jumping, accompanied by the rat running to the opposite end of the chamber) [52]. A mean shock reactivity score was calculated for each rat (i.e. the mean of the reactivity scores for all shocks received), and was used as a measure of pain sensitivity. The duration of burying was used as the primary measure of fear-related behaviour, with decreases in the duration of probe burying indicative of an attenuation of fear and anxiety, i.e. anxiolysis [79]. After each rat was tested any soiled bedding and all fecal boli were removed from the testing chamber. A layer of fresh bedding material was added and was smoothed to an even thickness approximately 4 cm in depth.

2.4.3. Shock-Probe Avoidance Retention

Five days after initial shock-probe testing, retention testing of shock-probe avoidance was conducted. Rats were tested in the same apparatus for 15 minutes or until first contact with the electrified shock-probe. No intracranial infusions were performed prior to retention testing. Latency to contact the shock-probe was measured and used to assess retention of shock probe avoidance learning; this latency measure has previously proved useful as an

assay of long term aversive conditioning [37]. Contact with the probe was operationally defined as contact sufficient for an electric shock to be delivered.

2.5. Histology

Upon completion of testing, rats were overdosed with chloral hydrate and perfused intracardially with 0.9% (wt/vol) saline followed by an infusion of 4% (vol/vol) formaldehyde. Subjects' brains were then removed and placed in a postfix solution of 4% formaldehyde for at least 48 hours. The brains were cut into 60 μm sections, and every second section was mounted onto glass slides and stained with thionin.

2.6. Statistical Analyses

The results from the elevated plus-maze test were expressed as means and standard errors of the mean (S.E.M.). Behavioural measures from the plus-maze were analyzed with one-way analysis of variance (ANOVA; α=0.05). Post hoc LSD tests were used to analyze the effects of drug treatment compared to the control condition (α=0.05). In order to correct for heterogeneity of variance and non-normality, the total time spent burying and immobile, measures recorded during shock-probe testing, were transformed to their natural logarithms (e.g. LOG [Total burying time +2]) prior to ANOVA testing (*the non-transformed means are shown in Table 6-1).* In addition, analysis of covariance (ANCOVA) was used to examine whether the effects of apomorphine on general activity

could be a possible confounding factor with respect to effects on fear-related behaviour. The plus-maze data of one animal was excluded because the rat failed to enter any of the arms during testing, and two rats were not tested in the shock-probe burying paradigm because their cannulae implants were unstable at the time of testing.

3. Results

3.1. Histology

Rats included in the analysis had infusion sites located in the prelimbic and infralimbic subregions of the MPFC. 15 rats had cannulae located outside of the prelimbic and infralimbic regions, and 5 rats showed substantial amounts of necrosis. Therefore, the behavioural results from these rats were excluded from the final data analysis. The approximate locations of the infusion sites for rats included in the data analysis are depicted in figure 1.

3.2. Elevated Plus-Maze Test

The results from plus-maze testing revealed that intra-MPFC infusions of apomorphine had a significant effect on the percentage of open-arm entries *($F(3,43)=2.79$, $P=0.05$; Figure 6-2a)*, and the percentage of open-arm time *($F(3,43)=3.37$, $P=0.03$; Figure 6-2b)*. Post hoc analysis revealed that the rats receiving either the 1 or 6 µg/Side doses of apomorphine showed a significantly higher percentage of open-arm entries, while rats receiving the 6 µg/Side

apomorphine dose also showed a higher percentage of time spent in the open arms when compared to control animals *(P≤0.05)*. However, apomorphine also affected the behavioural measures assessing general activity levels. Significant differences were found between the groups on the number of total arm entries *(F(3,43)=3.29, P=0.03; Figure 6-3a)*, and the number of closed arm entries *(F(3,43)=7.55, P=0.001; Figure 6-3b)*. Post hoc analysis revealed that the rats receiving either the 3 or 6 µg/Side doses of apomorphine showed a significantly lower number of total arm entries, while rats receiving any of the apomorphine doses showed a lower number of closed arm entries when compared to control animals *(P≤0.05)*. In order to examine whether the effects of apomorphine on these measures of general activity may have influenced the primary measures of anxiety in the plus-maze test, analysis of covariance (ANCOVA) was used with closed arm entries and total arm entries as the covariates. The ANCOVA indicated that the number of total arm entries and closed-arm entries (the covariates) did not account for the effects of apomorphine on open arm activity, since the differences between the treatment groups on the percentage of open-arm entries *(F(3,41)=2.96, P=0.04)* and the percentage time spent in the open arms *(F(3,41)=4.97, P=0.005)* were still significant.

3.3 Shock-Probe Burying Test

The results from shock-probe testing revealed that intra-MPFC infusions of apomorphine had a significant effect on the time the rats spent burying the probe *(F(3,42)=4.84, P=0.006; Figure 6-4; non-transformed means shown in*

Table 6-1). Post hoc analysis revealed that rats receiving the 6 µg/Side dose of apomorphine buried the probe significantly less than control animals *($P \leq 0.05$).* There were no significant differences between the groups on the number of shocks taken *($F(3,42)=0.29$, $P=0.84$; Table 6-1),* or shock reactivity *($F(3,42)=0.68$, $P=0.57$; Table 6-1).* However, there were significant differences between the treatment groups on time spent immobile during the shock-probe test *($F(3,46)=3.04$, $P=0.04$; Figure 6-5; non-transformed means shown in Table 6-1).* Post hoc analysis revealed that rats receiving the 6 µg/Side dose spent significantly more time immobile then controls *($P \leq 0.05$).* Once again, in order to examine whether the effects of apomorphine on general activity may have influenced the primary measure of anxiety, shock-probe burying, analysis of covariance (ANCOVA) was used with immobility time as a covariate. The ANCOVA showed that the effects of apomorphine on immobility (the covariate) may be responsible for the decrease in shock-probe burying, since the differences between the treatment groups on total time spent shock-probe burying were no longer significantly different when analyzed by ANCOVA *($F(3,41)=1.63$, $P=0.20$).*

3.4 Shock-Probe Avoidance Retention Test

The latency to contact the shock probe during retention testing was not significantly different across the treatment conditions *($F(1,35)=0.70$, $P=0.56$, Table 6-1)*, suggesting that intra-MPFC apomorphine did not influence retention of shock-probe avoidance behaviour.

4. Discussion

The results of the present study indicate that stimulation of medial prefrontal cortex (MPFC) dopamine (DA) receptors with the agonist apomorphine attenuated rats' fear-related behaviour in the elevated plus-maze test. Rats' behaviour in the shock-probe burying test was suggestive of apomorphine-induced anxiolysis, although the specific effects of apomorphine on burying behaviour could not be statistically separated from its effects on general activity. The findings from both tests showed that intra-MPFC infusions of apomorphine decreased measures of general activity.

In the elevated plus-maze, rats receiving bilateral intra-MPFC infusions of 1 or 6 μg of apomorphine had a significantly greater percentage of open-arm entries, and those receiving the 6 μg dose had a greater percentage of time spent in the open arms, when compared with control animals *(Figure 6-2a, 6-2b)*. Apomorphine also affected the two behavioural measures of general activity in this test, with the 3 and 6 μg doses decreasing the total number of arm entries, and all three doses decreasing the number of closed-arm entries *(Figure 6-3a, 6-3b)*. However, analysis of covariance (ANCOVA) revealed that the effects of apomorphine on the percentage of open-arm entries and time were still significant when the activity measures were treated as covariates, providing evidence that the apomorphine-induced attenuation of general activity was not

the primary causal factor underlying its ability to increase the percentage measures of open-arm activity. Overall, the results from elevated plus-maze testing suggest the intra-MPFC infusions of apomorphine, particularly the 6 μg dose, reduce the expression of fear-related behavioural responses (i.e. reduce avoidance of open arms).

In the shock-probe burying test, rats receiving intra-MPFC infusions of apomorphine spent significantly less time burying the shock-probe than controls *(Figure 6-4)*. However, as in the plus-maze, general activity appeared to decrease as a result of apomorphine treatment, with the 6 μg dose significantly increasing time spent immobile during shock-probe testing *(Figure 6-5)*. ANCOVA revealed that the decrease in general activity may have been an underlying causal factor behind the decline in burying behaviour. When the time spent immobile was used as a covariate, the effect of apomorphine on burying behaviour was not significant.

Although the ANCOVA indicated that drug effects on burying behaviour were confounded by drug effects on general activity, the analysis does not provide conclusive evidence that the apomorphine-induced inactivity caused the attenuation of shock-probe burying. In other words, it is still a possibility that the changes in general activity and burying behavior were two distinct effects both produced by the apomorphine treatment, as opposed to the decrease in general activity being responsible for the attenuation of the burying response. It is also important to note in this regard that an anxiolytic profile in the elevated plus-maze involves an increase in open-arm activity, while anxiolysis in the

shock-probe test is reflected by a decrease in probe-burying. Since significant anxiolytic-like effects were found in both tests, the overall pattern of these results would appear to argue against the findings simply being an artifact produced by attenuation of general activity levels, or by a non-specific inhibition or disinhibition of behavioural responses.

In contrast, other potentially confounding variables were not significantly affected by the infusions of intra-MPFC apomorphine. Pain sensitivity, as measured by shock reactivity, was not influenced by the apomorphine infusions. Short-term and long-term memory, as evaluated by the number of shocks taken during initial shock-probe testing, and latency to contact the probe during retention testing, respectively, were also unaffected by the DA agonist. These results suggest that the decrease in burying behaviour in the shock-probe test was not due to decreased sensitivity to the electric shock stimulus, or to an impaired ability to form an aversive association between the probe and the electric shock.

Nevertheless, the present findings do suggest that general activity was diminished by stimulation of MPFC DA receptors, as all activity measures decreased as a result of apomorphine treatment. This corroborates previous findings indicating that intra-MPFC infusions of DA agonists can produce inhibitory locomotor effects, while intra-MPFC DA antagonists can increase motor activity [2,19,34,54,81]. In summary, the findings taken as a whole suggest that stimulation of MPFC DA receptors with a nonspecific DA agonist is capable of attenuating fear-related behaviour and general activity.

The present results are particularly interesting given the research findings reported in previous studies. It has been reported that rats receiving intra-MPFC infusions of apomorphine show anxiogenic profiles in conflict procedures and pentylenetetrazol (PTZ) discrimination testing [6,7]. In contrast, infusions of the D1 agonist SKF-81297 have been reported to reduce anxiety-like behaviour in the elevated plus-maze test [82]. A separate study reported that both a DA agonist, D-amphetamine, and a DA antagonist, *cis*-flupenthixol, attenuated freezing behaviour to a conditioned cue, but did not appear to affect acquisition, suggesting *both* drugs produced anxiolytic-like profiles [53]. It is noteworthy that the studies reporting intra-MPFC application of a DA agonist induced anxiogenisis used conflict behaviour and PTZ discrimination to assess effects on fear and anxiety [6,7]. The MPFC is a crucial region in the neural mediation of reward, working memory and other cognitive processes [18,25,80]. Since both the conflict and PTZ discrimination procedures depend upon learning/memory and reward-related processes, the findings of these two studies may not reflect specific effects of the drug treatment on fear/anxiety. Therefore, interpreting the findings of these two intra-MPFC infusion studies can be viewed as problematic. In contrast, the studies reporting intra-MPFC treatment with DA agonists produce anxiolysis, including the present study, do not use behavioural assays with a reward component [53,82]. Furthermore, in the two studies examining the effects of intra-MPFC DA agonists on elevated plus-maze behaviour, an assay of anxiety without a memory/learning requirement, both studies found that DA agonists reduced fear-related behaviour [82]. Therefore, the existing data

appear to provide more compelling evidence to suggest that stimulation of MPFC DA receptors with a DA agonist produces an anxiolytic, as opposed to an anxiogenic, behavioural profile. Nevertheless, given the inconsistencies in the literature, further research into this issue should be conducted.

One general explanation for the diversity of findings reviewed above is that stimulation of MPFC DA receptors might produce unique effects on different types of fear behaviour. Therefore, it might be useful to systematically examine the effects of intra-MPFC apomorphine in different animal models of anxiety (e.g., those involving learned versus unlearned fear responses). Another potential explanation for the diverse findings is that stimulation or blockade of prefrontal DA receptors has different effects depending on the intensity of anxiety-provoking stimulus or baseline levels of DA activity. It is even possible that opposite effects may result from different levels of anxiety and/or levels of prefrontal DA.

Nevertheless, it seems counterintuitive that both stimulation and antagonism of MPFC DA receptors can produce anxiolysis. In light of these seemingly contradictory findings, it has been suggested that both stimulation and blockade may produce similar anxiolytic-like effects because there might be an optimal level of MPFC DA activity for the coordination of fear-related behaviour [53,82]. In other words, the robust expression of fear-related behaviour might require DA activity to be within a specific range, with both 'over-stimulation' and 'under-stimulation' of DA receptors within the MPFC disrupting or attenuating 'normal' fear responses. Studies investigating the effects of DA

agonists under different basal levels of anxiety might be useful in evaluating the merits of this hypothesis.

Intriguingly, the notion of MPFC DA effects having a type of inverted U-shaped function parallels the findings of research examining the effect of prefrontal DA on cognitive processes. Infusions of both DA agonists and antagonists into the prefrontal cortex have been shown to disrupt working memory, in rodents and primates, across a wide variety of behavioural assays [58,62-64,82-85]. These findings indicate that over- or under-stimulation of prefrontal DA can disrupt working memory, suggesting existence of an optimal level of DA stimulation. It is possible that the same notion can be applied to fear and anxiety.

That prefrontal DA may influence both anxiety and working memory raises some interesting issues. One concerns the extent to which different functions affected by MPFC DA might influence each other via the same system. Previous research has already shown that increases in prefrontal DA evoked by anxiogenic stimuli are capable of producing deficits in working memory, and this effect can be reversed by treatment with dopamine antagonists [1,45,46]. In light of this evidence, further investigation of possible prefrontal DA mediated interactions between cognitive and emotional processes may prove to be fruitful. Another issue concerns whether MFPC DA actually affects some underlying function or process, which in turn influences fear/anxiety and working memory. For example, it is possible that the impairment in emotion and memory could result from a more fundamental impairment in behavioural flexibility. Previous

research has shown that the MPFC is important for the expression of behavioural flexibility inherent in shifting strategies [3,17,55,56]. Therefore, inappropriate behavioural responses with respect to fear/anxiety, and memory, may be a reflection of an inability to successfully shift behavioural repertoires in response to changing contexts. While such interpretations are interesting and warrant further examination, at present they are highly speculative.

Apomorphine is a general DA agonist without high selectivity for any specific DA subtype [65]. Therefore, the present findings provide no information on the effects of stimulation of selective DA receptor subtypes. Since previous work has shown that selective blockade of MPFC D4 receptors attenuates fear-related behaviour [66], it would be of particular interest to examine the effects of a selective D4 agonist on fear behaviour. Another promising area of research involves the role of MPFC norepinephrine and serotonin in fear and anxiety. It has been shown that in addition to dopamine, MPFC norepinephrine and serotonin activity also increase as a result of exposure to fear-provoking stimuli [24,28,29,47]. However, at present we are not aware of any study investigating effects of stimulation or blockade of prefrontal serotonin or norepinephrine receptors on fear behaviour. Such studies would further enhance our understanding of how the MPFC mediates fear, anxiety, and stress.

Figure 6-1. Diagram of coronal rat brain sections with markings illustrating the approximate location of internal infusion cannulae (specifically, the most ventral point of the cannulae) for all animals included in the present study. Cg1= Anterior cingulate area; DP= Dorsal peduncular area; IL= Infralimbic area; PrL= Prelimbic area; M2= Frontal Cortex, Area 2; VO= Ventral orbital area. [49].

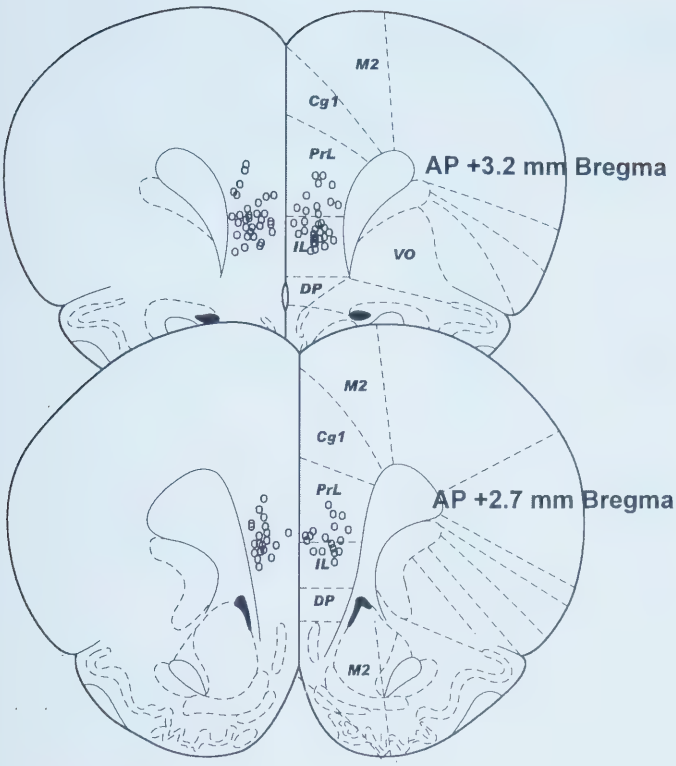

M2
Cg1
PrL
AP +3.2 mm Bregma
IL
VO
DP
M2
Cg1
PrL
AP +2.7 mm Bregma
IL
DP
M2

Figure 6-2. **(A)** Mean (±S.E.M.) percent open arm entries during elevated plus-maze. **(B)** Mean percent open arm during elevated plus-maze testing. Four different treatment groups are shown in this figure: bilateral intra-MPFC infusions of Vehicle (distilled water), Apomorphine (1 µg/0.5 µl), Apomorphine (3 µg/0.5 µl), Apomorphine (6 µg/0.5 µl). * P<0 .05 for comparisons between the treatment group and control animals. ** P<0 .01.

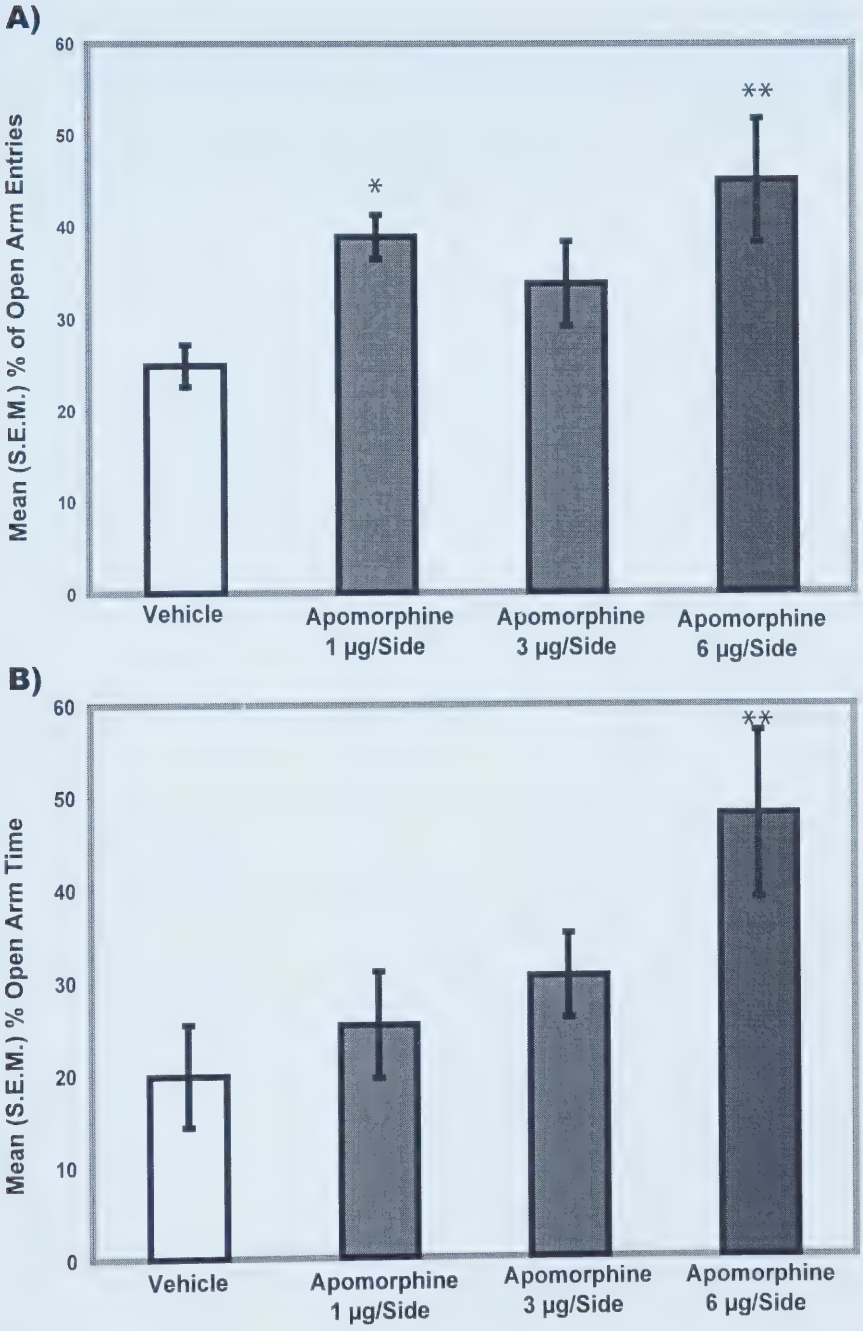
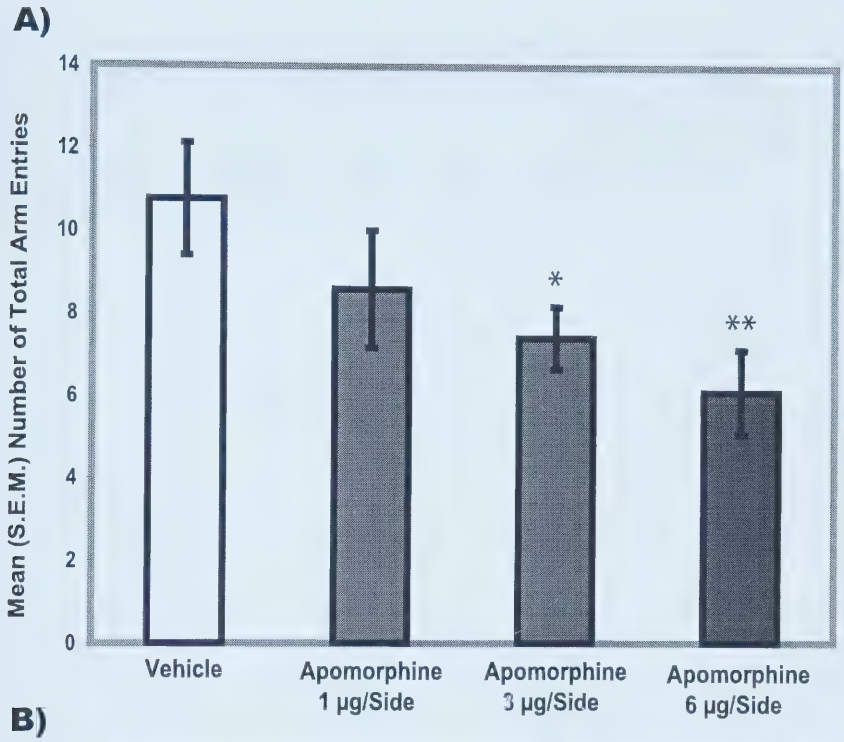

A)
Mean (S.E.M.) % of Open Arm Entries
60
50
40
30
20
10
0
*
**
Vehicle
Apomorphine 1 µg/Side
Apomorphine 3 µg/Side
Apomorphine 6 µg/Side
B)
Mean (S.E.M.) % Open Arm Time
60
50
40
30
20
10
0
**
Vehicle
Apomorphine 1 µg/Side
Apomorphine 3 µg/Side
Apomorphine 6 µg/Side

Figure 6-3. **(A)** Mean (±S.E.M.) total number of all arm entries during elevated plus-maze. **(B)** Mean total number of closed arm entries during elevated plus-maze testing. Four different treatment groups are shown in this figure: bilateral intra-MPFC infusions of Vehicle (distilled water), Apomorphine (1 μg/0.5 μl), Apomorphine (3 μg/0.5 μl), Apomorphine (6 μg/0.5 μl). * P<0 .05 for comparisons between the treatment group and control animals. ** P<0 .01.


A)
Mean (S.E.M.) Number of Total Arm Entries
14
12
10
8
6
4
2
0
*
**
Vehicle
Apomorphine 1 µg/Side
Apomorphine 3 µg/Side
Apomorphine 6 µg/Side

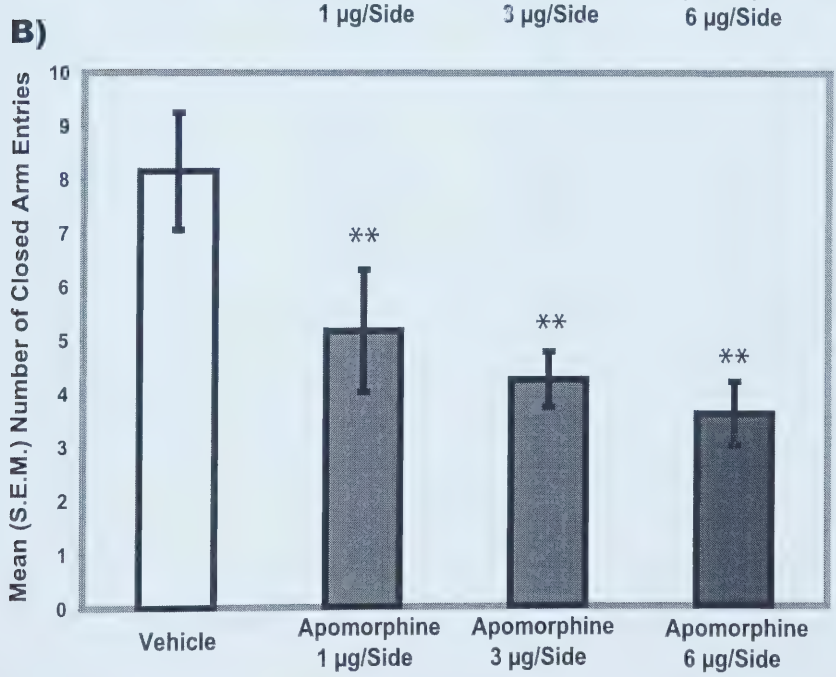

B)
Mean (S.E.M.) Number of Closed Arm Entries
10
9
8
7
6
5
4
3
2
1
0
**
**
**
Vehicle
Apomorphine 1 µg/Side
Apomorphine 3 µg/Side
Apomorphine 6 µg/Side

Figure 6-4. Mean (±S.E.M.) time (in seconds) spent burying the shock-probe. All means shown have been logarithmically transformed. Four different treatment groups are shown in this figure: bilateral intra-MPFC infusions of Vehicle (distilled water), Apomorphine (1 µg/0.5 µl), Apomorphine (3 µg/0.5 µl), Apomorphine (6 µg/0.5 µl). * P<0 .05 for comparisons between the treatment group and control animals. ** P<0 .01.

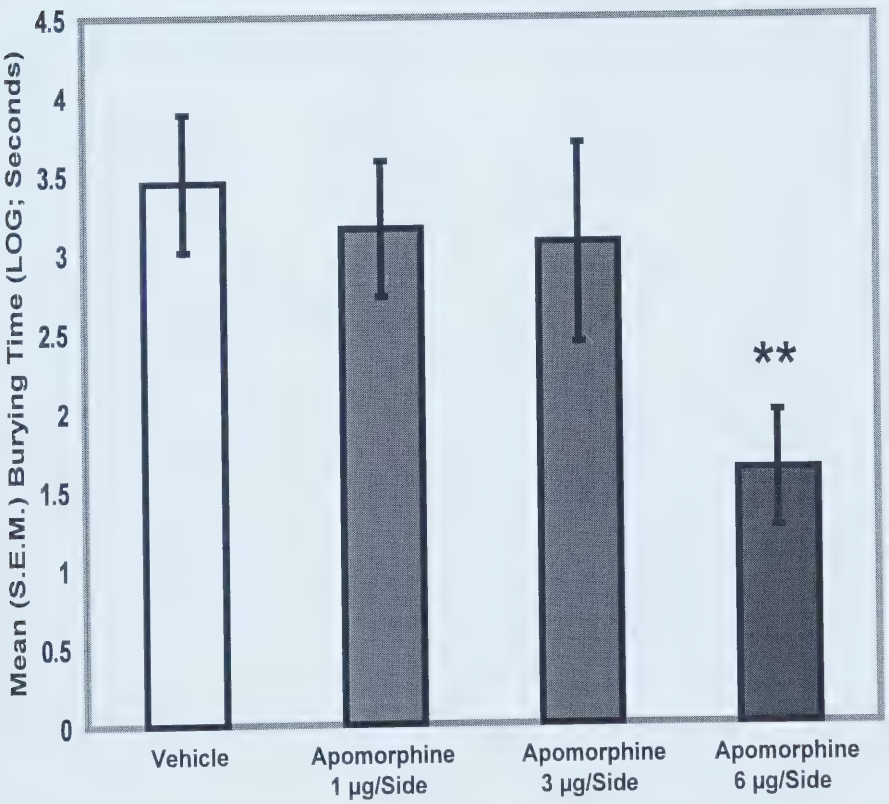

Mean (S.E.M.) Burying Time (LOG; Seconds)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
Vehicle
Apomorphine 1 µg/Side
Apomorphine 3 µg/Side
Apomorphine 6 µg/Side

Figure 6-5. Mean (±S.E.M.) time (in seconds) spent immobile (primarily rest or sleep). All means shown have been logarithmically transformed. Four different treatment groups are shown in this figure: bilateral intra-MPFC infusions of Vehicle (distilled water), Apomorphine (1 µg/0.5 µl), Apomorphine (3 µg/0.5 µl), Apomorphine (6 µg/0.5 µl). * P<0 .05 for comparisons between the treatment group and control animals. ** P<0 .01.

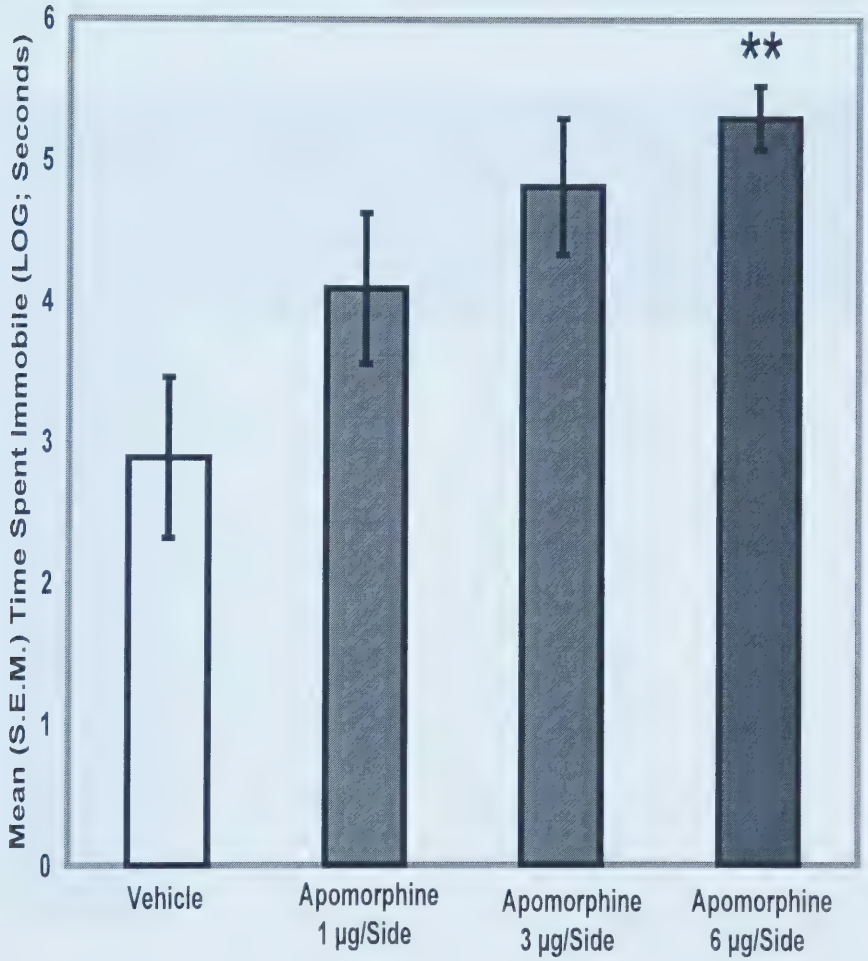

Mean (S.E.M.) Time Spent Immobile (LOG; Seconds)
0
1
2
3
4
5
6
**
Vehicle
Apomorphine 1 µg/Side
Apomorphine 3 µg/Side
Apomorphine 6 µg/Side

Table 6-1. Mean (±S.E.M.) untransformed scores for behavioural measures recorded during the shock-probe burying test.

Treatment/ Group	Shock-Probe Buring Time (in Seconds)	Immobility Time (in Seconds)	Number of Shocks Received	Mean Shock Reactivity Score	Latency to Contact Probe (in Seconds)
Vehicle-Control	**69** (±22.6) *n= 13*	**81.5** (±34.7) *n= 13*	**1.9** (±0.3) *n= 13*	**2.3** (±0.2) *n= 13*	**500.1** (±100.6) *n= 13*
Apomorphine *(1 µg/ Side)*	**53.3** (±23.8) *n= 12*	**146.4** (±43.1) *n= 12*	**1.9** (±0.2) *n= 12*	**2.1** (±0.1) *n= 12*	**340.3** (±96.7) *n= 11*
Apomorphine *(3 µg/ Side)*	**65.5** (±23.0) *n= 10*	**231.9** (±59.1) *n= 10*	**1.6** (±0.2) *n= 10*	**2.1** (±0.1) *n= 10*	**390.6** (±108.0) *n= 10*
Apomorphine *(6 µg/ Side)*	**8.8** (±4.8) *n= 11*	**244.6** (±44.9) *n= 11*	**1.8** (±0.3) *n= 11*	**2** (±0.2) *n= 11*	**301.2** (±90.2) *n= 11*

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Chapter 7

General Discussion

1. Overview of Findings and Implications

The present series of studies suggest that the medial prefrontal cortex (MPFC), particularly MPFC GABA and dopamine (DA) activity, has a significant role in the neural mediation of fear and anxiety-related behaviour (see summary of findings in Table 7-1). The first study revealed that excitotoxic lesions of the MPFC decreased fear-related behavioural responses in the elevated plus-maze, social interaction, and shock-probe burying tests (Chapter 2). Lesions increased open-arm activity and the duration of active social interaction, and decreased burying behaviour. The lesions did not affect measures of aversive memory (number of shocks taken; retention of shock-probe avoidance), pain sensitivity (shock reactivity), or general activity (number of closed-arm entries; time spent immobile), making it unlikely that lesion effects on these processes could have accounted for the effects on fear-related behavior. These findings provide clear evidence that excitotoxic lesions of the MPFC selectively attenuate a wide variety of fear-related behavioural responses, and suggest a significant role for the MPFC in the neural mediation of fear behaviour.

The second and third studies examined the effects of intra-MPFC infusions of indirect and direct $GABA_A$ receptor agonists on fear behaviour. The results of the second study showed that intra-MPFC infusions of the benzodiazepine midazolam (5 μg/μl), an indirect $GABA_A$ agonist, produced anxiolytic-like effects in the elevated plus-maze and shock-probe burying tests.

These effects appeared to be independent of the dorsal or ventral placement of midazolam infusions within the MPFC (Chapter 3). The third study extended these findings by showing that intra-MPFC infusions of the direct $GABA_A$ agonist muscimol (0.175 nmol or 4 nmol/0.5 µl) also increased open-arm activity and decreased burying behaviour (Chapter 4). Both studies also showed that intra-MPFC infusions of these $GABA_A$ agonists did not affect general activity, pain sensitivity, or avoidance learning, reinforcing the behavioral specificity of the results. Additionally, in neither study did animals with misplaced cannulae show anti-fear effects, suggesting the site-specificity of the results. Overall, these findings indicate that increased GABAergic inhibition within the MPFC produces clear-cut anxiolytic effects.

It is noteworthy that the highest dose of muscimol (4 nmol/0.5 µl), which was anxiolytic in both the plus-maze and shock-probe tests, is comparable to doses that have previously been used to produce neural inactivation of a region [18,81]. Thus it appears that disabling the MPFC, either by excitotoxic lesions (Chapter 2) or by GABA-induced inactivation (Chapter 4), attenuates fear-related behavior in the rat. This pattern of results does not appear to be consistent with the notion that the MPFC influences fear and anxiety by exerting inhibitory control over other regions, such as the amygdala. If the MPFC did have a simple inhibitory role, one would expect that disabling the MPFC would actually *enhance* the expression of fear and anxiety. Since it was shown that lesions or pharmacological inactivation of the region actually *decreased* rats' fear-related responses, the present findings do not appear to agree with a simple

inhibitory model of the MPFC. Instead, they lend support to a broader view of MPFC function, one that includes a role for the region in the activation, or basic coordination, of fear-related behaviour, and not just inhibition of these responses. It is important to note, however, that the present findings do not preclude the involvement of the MPFC in inhibitory processes such as the extinction of fear conditioning. There is substantial evidence that the MPFC has a role in processes related to the extinction of conditioned fear, and furthermore, is capable of inhibiting amygdala activity related to fear conditioning [30,35,36,38-41,47-49,58]. These findings have inspired a conceptualization of the MPFC as a primarily inhibitory center with respect to fear and anxiety. Overall, however, the present results suggest that the MPFC does not exclusively play an inhibitory role in the mediation of fear-related behaviour, but instead also contributes to the expression of fear responses.

The fourth and fifth studies investigated the involvement of MPFC DA in fear-related behaviour. The results of the fourth study showed that intra-MPFC infusions of the highly selective D4 antagonist L-745,870 increased open-arm activity (1 nmol/0.5 µl), and decreased the duration of burying (0.2 or 1 nmol/0.5 µl), while the D1 (SCH-22390) and D2 (Remoxipride) antagonists had no significant behavioural effects (Chapter 5). Intra-MPFC infusions of the D4 antagonist did not affect general activity, pain sensitivity, or shock-probe avoidance. In addition, fear-related measures were not significantly affected in animals with misplaced cannulae. Overall, these findings indicate that blockade of MPFC D4 receptors significantly and selectively inhibit fear behaviour. The

fifth study showed that intra-MPFC infusions of the non-subtype-selective DA agonist, apomorphine, increased open-arm activity (1 and 6 μg/0.5 μl), and decreased burying behavior (6 μg/0.5 μl), but also decreased measures of general activity (Chapter 6).

Taken together, the findings from studies four and five suggest that MPFC DA receptors substantially influence fear-related behaviour. However, they also provide a seemingly inconsistent pattern of findings since both a general DA agonist (apomorphine) and a selective D4 antagonist (L-745,870) attenuated fear behaviour. There is not a clear explanation for why both a DA agonist and a DA antagonist would produce anxiolytic-like effects in the elevated plus-maze and shock-probe burying tests. However, previous intra-MPFC DA receptor agonist/antagonist infusion studies have also revealed what appears to be a complex pattern of findings [8,9,44,77,78]. One explanation for the present findings is that apomorphine decreased general activity rather than fear and anxiety. While this possibility may explain the decrease found in burying behaviour, it does not seem to account for the increase found in open-arm activity, which was significant even after between-group variation in general activity had been statistically controlled (ANCOVA). Another potential explanation is that there are differential effects on fear behaviour resulting from stimulation/inhibition of specific DA receptor subtypes. Since apomorphine is a general DA agonist, effects at non-D4 receptor sites may have accounted for its anxiolytic-like effects. According to this view, a selective D4 agonist might be expected to increase fear-related behaviour, whereas a non-subtype-selective

agonist, such as apomorphine, might produce an opposite effect. While differential effects mediated through specific DA receptor subtypes may be a factor, such an explanation does not adequately account for the complexity of the current literature. For example, previous findings have indicated that intra-MPFC infusions of a subtype-selective D2 agonist *and* antagonist both produce anxiolytic-like effects [78]. Another possible explanation is that the anxiolytic-like effects of apomorphine were actually caused by a decrease in MPFC DA activity because the DA agonist primarily exerted effects at DA autoreceptors, which in turn *decreased* DA activity. However, such an explanation is also problematic since DA autoreceptors appear to have minimal influence over the regulation of prefrontal dopamine activity [59]. Perhaps the most credible explanation for the current set of findings is that fear-related behaviour may require DA activity to be within a specific range, with both 'over-activity' and 'under-activity' of the MPFC dopaminergic system disrupting the normal behavioural expression of fear and anxiety. This would parallel findings relating to the role of MPFC DA and working memory [54,61-63,77,78,82,83]. On the other hand, it is generally atypical for an agonist and antagonist of the same receptor site to generate similar effects. In addition, the present findings do not appear entirely consistent with such an explanation as the 1 nmol/0.5 µl dose of the selective D4 antagonist L-745,870 consistently affected fear-related behaviour, while the 10 nmol/0.5 µl dose did not significantly affect open-arm activity or burying (Chapter 5). These findings do not appear to be in agreement with the argument that disrupting an optimal level of DA activity produces

anxiolytic-like effects; i.e. the highest dose. which should have produced the highest level of D4 receptor blockade, did not significantly affect fear-related behaviour. In conclusion, the mechanisms that might account for MPFC DA receptor effects on fear behaviour are unclear. Further research is required in order to more fully characterize and elucidate the effects of MPFC DA activity on fear behaviour.

Another curious aspect of the present findings is the apparent inability of neurochemical manipulation or lesioning of the MPFC to produce effects on measures related to conditioned fear in the shock-probe test. No experimental treatment in the present series of studies significantly affected the number of shocks taken during shock-probe testing, a measure that has been used to assess aversive memory [32,33]. In addition, retention of shock-probe avoidance was not affected by MPFC lesioning or intra-MPFC infusions of apomorphine (Chapters 2 and 6). In contrast, other fear-related behaviour in the elevated plus-maze and shock-probe burying tests were significantly affected by a variety of experimental manipulations. It is important to remember, however, that the behavioral tests used in the present experiments were explicitly designed to study rats' untrained or unconditioned fear reactions, rather than conditioned fear reactions. Thus they may be inherently less sensitive to effects on rats' conditioned fears.

Within the area of the neurobiology of anxiety, the amygdala has been the focus of a vast amount of research (e.g.[14,25,30,31,47,52]). Given that research in the area has often primarily concentrated on the amygdala, it might

be expected that the influence of other brain regions on fear and anxiety has gone relatively unexamined. While the amygdala likely has a central role in the mediation of certain fear-related responses, there is evidence that other regions also substantially contribute to fear-related behavior. For example, septal lesions and microinfusions of anxiolytic drugs targeting the lateral septum were shown to produce anxiolytic-like effects in the shock-probe and elevated plus-maze tests [34,42,43,71]. In contrast, amygdala lesions do not typically affect open-arm activity [26,65,71,72], or burying behaviour [27,57,70-72]. As described previously (Chapter 1), extensive research has indicated that the MPFC is involved in mediating the physiological, endocrine, and neurochemical correlates of fear and anxiety. The present findings contribute to the body of evidence suggesting that the MPFC is also important for the mediation of essential fear-related behaviour.

While current evidence clearly implicates the MPFC in fear and anxiety, the precise way in which it serves this function is open to question. The issue of whether the MPFC has a primary, or a secondary modulatory role (e.g. modulation of the amygdala or the septohippocampal system), with regards to fear behaviour is not clear. Further investigation is necessary to determine the extent to which the MPFC exerts effects directly on structures mediating the components of fear-related responses, or through intermediary structures that are primarily responsible for coordinating these fear-related responses. The MPFC may indirectly influence fear behaviour through processes that contribute to the general organization of cognition and behaviour, on which the expression of

fear-related responses is dependent. For example, previous research has shown the MPFC is important for the expression of behavioural flexibility [1,15,50,51]. It is possible that impairment in the shifting of behavioural sets in response to a changing environment results in what appears to be a simple anxiolytic effect. Another general interpretation has been provided by Antonio Damasio, who has argued that the prefrontal cortex is responsible for the integration of somatic markers of emotion, physiological markers that are important for informing any decision-making dependent on emotional information [13]. The present data could be interpreted within the context of, and viewed as complementary to, such a model. The anxiolytic-like effects produced by experimental manipulation of the MPFC may have resulted from impairment in the integration of emotional input (i.e. integration of somatic markers) necessary for informing controlled responses related to fear and anxiety. More generally, the present findings are in accord with the notion that one of the fundamental functions of the prefrontal cortex is to integrate behavioural responses related to emotion. Regardless of the precise underlying processes mediated by the MPFC, given the corpus of evidence that presently exists, it seems reasonable to include fear and anxiety on the list of functions mediated in part by the MPFC.

2. Limitations of the Present Research

One limitation of the present findings concerns the absence of pharmacological controls for the intracranial infusion studies . Specifically, the

receptor selectivity of midazolam or muscimol was not confirmed by reversing the anxiolytic-like effects of these $GABA_A$ agonists with a sub-effective dose of a $GABA_A$ antagonist, such as a β-carboline or bicuculline. However, since the selective effects of muscimol and midazolam on the $GABA_A$ receptor complex are well established, it is highly probable that the anxiolytic-like effects produced by intra-MPFC infusions of these agonists primarily resulted from agonistic action at $GABA_A$ receptors. Similarly, it was not shown that the anxiolytic-like profile produced by the D4 antagonist, L-745,870, could be reversed by treatment with a D4 agonist. On the other hand, L-745,870 has been shown to be a highly selective D4 antagonist (> 2000-fold affinity over other DA receptors [7]). Therefore, it is likely that the decrease in fear-related behaviour produced by L-745,870 did in fact result from blockade of D4 receptors.

The absence of effects produced by the selective D1 and D2 antagonists is also curious. It must be noted that these results do not conclusively demonstrate that selective D1 or D2 antagonism is not capable of significantly affecting fear behaviour. It is possible that greater (or lesser) levels of D1 or D2 blockade would have produced significant effects on fear-related behaviour. In particular, there is evidence to suggest that blockade of MPFC D2 receptors can produce anxiolysis, since intra-MPFC infusions of the D2 antagonist sulpiride produced anxiolytic-like effects in the murine elevated plus-maze [78]. Nonetheless, it should be noted that a reasonably broad range of doses (0.2, 1, 10 nmol, Remoxipride; 0.2, 1 nmol, SCH-23390) was used in the study described in Chapter 5. On the whole, the findings on D1 and D2 antagonists should be

viewed as preliminary; additional research is necessary to fully elucidate the influence of specific DA receptor subtypes on fear and anxiety.

The present research also has some limitations with regard to the types of fear-related behaviour that were examined. For example, the influence of the MPFC on the defensive behaviour provoked by predatory stimuli could have been assessed utilizing well-established paradigms [3-6,16,79]. Additionally, assays specifically designed to assess conditioned fear were not used in the present study, and the extinction of conditioned fear was not examined. While the present studies did not examine all aspects of fear-related behaviour, these studies still provided a behaviourally rigorous examination of the MPFC. Three different well-validated behavioural assays were utilized, and behavioural controls were used to rule out a variety of possible confounding effects.

Another issue is the generalizability of the present findings, given the substantial differences that exist between the rat and human prefrontal regions. The prefrontal cortices of different mammalian species show considerable anatomical variation. In humans, this region is unusually well developed; it is the most extensive cortical region, occupying approximately 30% of the cerebral cortex (As a comparative example the prefrontal region represents 3.5% of the cortex in cats) [20,75]. In addition to differences in size, the rat cortex also differs from the primate cortex by being lysencephalic, less segregated and differentiated, and with no well-developed internal granular layer [45,74,84]. However, it has been argued that anatomical connectivity is likely more useful than cytoarchitectonic features, since there are numerous cytoarchitectonic

differences that exist between species, and even individuals of the same species [20]. There are in fact considerable fundamental similarities between the prefrontal regions of rodents and primates in terms of anatomical connectivity and function. Both the rat and primate prefrontal regions have similar patterns of connectivity with the mediodorsal thalamus, basal ganglia, and brainstem nuclei. The rat and primate prefrontal regions also share an array of functional similarities such as emotion, working memory, and cognitive flexibility [74]. With regard to specific regions of the prefrontal cortex, probably the most contentious issue is whether the rat prefrontal cortex contains a region homologous to the primate dorsolateral area (there appears to be far more agreement on the existence of homologous medial and orbital regions in the rat) [45,46,74]. The rat MPFC itself has been argued to have similarities with the primate anterior cingulate, orbital, and dorsolateral prefrontal regions [45,74,76]. Given the relatively undifferentiated nature of the rat prefrontal cortex, it may be the case MPFC is an amalgam of areas located in the primate anterior cingulate, orbital, and dorsolateral regions. Regardless, the consensus appears to be that rat prefrontal cortex is generally homologous to a broadly defined primate prefrontal cortex. However, given the anatomical differences, all of the findings related to the rat prefrontal cortex may not be entirely generalizable to humans. Therefore, corroborating the findings of rodent studies with primate or human research is an important task.

3. Future Research Directions

Given the complex body of findings that presently exists (Chapter 5 and 6; [8,9,44,77,78]), further research is necessary in order to fully characterize the effects of MPFC DA on fear behaviour. As mentioned above, one possible explanation for anxiolytic-like effects produced by both intra-MPFC infusions of DA agonists and antagonists is that a specific range of DA activity is necessary for the optimal expression of fear behaviour. In order to evaluate the validity of this hypothesis, it would be useful to collect more data on the effects of intra-MPFC infusions of DA agonists/antagonists on fear-related behaviour- over a wide range of doses. The hypothesis would predict that relatively high doses of DA receptor agonists or antagonists would attenuate fear-related behaviour, while lower doses would have no effect or possibly increase fear-related responses. Examining the effects of intra-MPFC infusions of different doses of DA agonists and DA antagonists in the same experiment might prove to be particularly insightful, since such a design can directly evaluate whether both DA stimulation or DA blockade are capable of producing anxiolytic-like effects. One approach would be to combine in vivo DA assay techniques, such as voltammetry, intra-MPFC DA treatment, and assays of fear-related behaviour. Such a combination of techniques could be utilized in the following manner: 1. DA activity in the MPFC would be recorded with fine temporal resolution using in vivo voltammetry. 2. Animals could then be given intra-MPFC infusions of DA agonists/antagonist at different basal levels of DA activity, and the subsequent effects on fear-related responses could be assessed. The optimal DA

range hypothesis would predict that a DA agonist would produce anxiolytic-like effects when MPFC DA levels are high, while a DA antagonist would produce similar effects when MPFC DA levels are relatively low.

The effects of DA agonists/antagonists on DA autoreceptors may also be an underlying mechanism behind the complex set of intra-MPFC DA treatment findings. Therefore, it would be of interest to explore the role of MPFC DA autoreceptors on fear-related behaviour. This research objective could be accomplished by investigating the effects of intra-MPFC infusions of antagonists/agonsists having preferential action at DA autoreceptors, compounds such as (+)-AJ 76 or (+)-UH 232 [66]. If DA autoreceptor effects account for the anxiolytic-like effects of intra-MPFC apomorphine, then blockade of DA autoreceptors should produce an anxiogenic-like profile. Furthermore, the effects of apomorphine should be able to be reversed by the DA autoreceptor antagonists.

More data on the effects of selective stimulation and/or blockade of various DA receptor subtypes, would also be helpful in elucidating the role of MPFC DA on fear behaviour. At present only a few studies have examined the effects of intra-MPFC infusions of DA receptor subtype-specific agonists/antagonists on fear-related behaviour [64,77,78]. Investigation of MFPC D3 receptors might be of particular interest. The effects of selective MPFC D3 receptor stimulation, or blockade, on fear-related responses have yet to be examined. There is also evidence to suggest D3 compounds may influence fear-related behaviour [55,56], and activity in the prefrontal cortex [2].

A systematic examination of the effects of fear-provoking stimuli of varying intensities on MPFC DA activity would also enhance our understanding of this system. While there is a vast amount of data indicating MPFC DA activity increases in response to fear-provoking stimuli [10-12,22,23,68,69], the effects of intensity of fear/stress on the MPFC DA response has not been fully characterized. It has been shown, however, that selective activation of the mesoprefrontal DA specifically occurs during mild or moderate levels of fear/stress [23,28,60]. Given these findings, examining the effects of DA receptor blockade and stimulation under different basal levels of fear or stress may also provide interesting information on whether MPFC DA responds differentially depending on the intensity of fear/stress.

Another avenue for future research is the investigation of possible interactions between MPFC dopaminergic and GABAergic systems, with regard to effects on fear and anxiety. There is evidence suggesting that DA activity is capable of modulating GABAergic inhibitory signaling in the MPFC [21,29,80]. Furthermore, $GABA_A$ agonists are known to inhibit MPFC DA responsivity to various stressors [17,19,24,28,67], and have been shown to attenuate fear-related behaviour when infused into the MPFC (Chapter 3 and 4). These various findings suggest that interactions between MPFC DA and GABA may significantly influence fear behaviour. One way of examining this would be to see if sub-effective doses of various DA compounds are capable of reversing or enhancing the effects of selective $GABA_A$ compounds. A reversal or enhancement would provide evidence for an interaction between the MPFC

GABA and DA systems. Another way of investigating this issue would be to combine intra-MPFC microinfusions with in vivo assays of GABA activity (e.g. microdialysis, voltammetry). Such an experimental design could be used to reveal the effects of fear/stress on MPFC GABAergic activity, and also used to assess if MPFC DA activity modulates GABAergic responses evoked by fear-provoking stimuli.

A more expansive line of inquiry involves investigating the role of the mesoprefrontal DA system in mediating a *range* of emotions. While there is evidence to suggest that MPFC DA is important for the expression of fear behaviour, there is also evidence indicating that the MPFC DA has a role in mediating reward-related behaviour (e.g. [37,53,73]). Previous research has focused on either fear or reward separately. Fear and reward have been compartmentalized, and their distinct lines of research have not been adequately integrated. Since the mesoprefrontal DA system has been shown to be important for both functions, examining both emotional states simultaneously will likely prove valuable. Specifically, it would be informative to examine the patterns of DA activity in the MPFC, and nucleus accumbens (NAc) (given the role of this region in mediating reward), in response to fear, reward, and a combination of the two states. This objective could be accomplished by investigating MPFC and NAc DA activity in response to the Geller-like conflict procedures. These tests examine reward behaviour (food or water rewards), and conflict behaviour (combination of rewarding and aversive stimuli) during trials in which the reward is accompanied by an electric shock. Changes in DA levels in response to

the fear-inducing stimulus (electric shock) alone would be assessed in a separate group. This research will provide a comparison of the pattern of dopamine activity in response to aversive, rewarding, and emotionally conflicting stimuli, within the same experiment, and would help to clarify the contribution of MPFC and NAc DA on the neural mediation of fear and reward states. More generally, a careful and systematic program of research which aims to characterize mesolimbocortical DA across varying conditions of fear/anxiety and pleasure/reward (i.e. across a continuum of emotional states) holds the promise of providing valuable data which may enhance our understanding of how the brain mediates emotion.

Table 7-1. Summary of Findings.

Experimental Treatment	Elevated Plus-Maze	Shock-Probe Burying	Other Results
MPFC Lesioning	**Increased Open-arm Activity**	**Decreased Burying**	**Increased Active Social Interaction** **No Effects:** *SP- Number of Shocks and Retention, General Activity, Pain*
Midazolam *(5 μg)*	**Increased Open-arm Activity**	**Decreased Burying**	**No Effects:** *SP- Number of Shocks, General Activity, Pain*
Muscimol *(0.175 nmol)*	**Increased % Open Time**	**Decreased Burying**	*Increased General Activity* **No Effects:** *SP- Number of Shocks, Pain*
Muscimol *(4 nmol)*	**Increased Open-arm Activity**	**Decreased Burying**	*Increased General Activity* **No Effects:** *SP- Number of Shocks, Pain*
Remoxipride *(0.2 nmol)*	**No Effect**	**No Effect**	
Remoxipride *(1 nmol)*	**No Effect**	**No Effect**	
Remoxipride *(10 nmol)*	**No effect**	**No Effect**	
SCH-23390 *(0.2 nmol)*	**No Effect**	**No Effect**	
SCH-23390 *(1 nmol)*	**No Effect**	**No Effect**	
L-745,879 *(0.2 nmol)*	**No Effect**	**Decreased Burying**	**No Effects:** *SP- Number of Shocks, General Activity, Pain*
L-745,870 *(1 nmol)*	**Increased Open-arm Activity**	**Decreased Burying**	**No Effects:** *SP- Number of Shocks, General Activity, Pain*
L-745,870 *(10 nmol)*	**No Effect**	**No Effect**	
Apomorphine *(1 μg)*	**% Open-arm Entries**	**No Effect**	*Decreased General Activity* **No Effects:** *SP- Number of Shocks, Pain*
Apomorphine *(3 μg)*	**No Effect**	**No Effect**	*Decreased General Activity* **No Effects:** *SP- Number of Shocks, Pain*
Apomorphine *(6 μg)*	**Increased Open-arm Activity**	**Decreased Burying**	*Decreased General Activity* **No Effects:** *SP- Number of Shocks, Pain*

Notes: All intra-MPFC infusions were bilateral. 'Increased Open-arm Activity' represents increased % Open-arm Entries and Time. 'SP' = Shock-Probe Burying Test.

3. References

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